BIOLOGICAL ACTION OF PROPROTEIN CONVERTASE PC1 IN HUMAN BREAST CANCER

by

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ABSTRACT

Proprotein convertases have been proposed to be critical enzymes important in the progression of breast cancer, based on their unique property of generating biologically active growth and receptor molecules from their inactive precursors. In order to understand the biological action of proprotein convertases in the human breast cancer cell line, MCF-7 was stably transfected with a CMV-mPC1 / neo construct. Eight G418 resistant clones were tested by northern blot and Southern blot; only clone 2 and clone 6 were found to express mPC1 mRNA. Clone 6, as a higher expressor of mPC1 mRNA level compared to clone 2, was labeled with ^{35}S-Cysteine. Twenty-four hours after labeling, both cell lysate and medium were collected. Immunoprecipitation with rabbit polyclonal anti-PC1 antibody followed by one- and two-dimensional SDS-polyacrylamide gel electrophoresis was carried out, to confirm that an elevated level of mPC1 protein was produced in the stable transfected cell line. In order to identify potential PC1 target proteins in human breast cancer, two-dimensional gel analysis was used to compare cellular proteins between clone 6 and clone 7; the latter was a G418 resistant clone but did not contain an integrated mPC1 construct. After four separate experiments, four consistent and reproducible proteins were identified which differed between clone 7 and clone 6. One or more of these proteins may be a candidate for PC1 cleavage in human breast cancer. Further analysis of these protein spots is necessary to gain insights into the identity and potential relationship between these proteins.

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
α_1 -PDX	α_1-antitrypsin Portland
BBS	N, N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid and buffered saline
CMV	Cytomegalovirus
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGF / EGFR	Epidermal growth factor / EGF receptor
ER	Estrogen Receptor
EtBr	Ethidium Bromide
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
GRB	Gel running buffer
HGF	Hepatocyte growth factor
HUVEC	Human umbilical vein endothelial cells
IAPP	Islet ancyloid polypeptide
IGF	Insulin-like growth factor
Kb	Kilo base
Kda	Kilo Dalton
LPH	Lactase-phlorizin hydrolase
MAP	Mitogen activated kinase
MDGF-I	Mammary-derived growth factor
MMP	Matrix metalloprotease
MMTV	Mouse mammary tumor virus
mRNA	messenger RNA
Na / NaCl / NaOH / NaAc	sodium /-chloride /-hydroxide /-acetate
NEO	Neomycin
NP-40	nonidet-40
PA	Protective Antigen
PBS	Phosphate-Buffered Saline
PC	Proprotein convertase
PCR	Polymerase Chain Reaction
PDGF	Platelet-Derived Growth Factor
PKC	Protein Kinase C
POMC	Proopiomelanocortin
PPO	2,5-diphenyloxazole

PR	Progesterone Receptor
RPM	revolutions per minute
SCLC	Small Cell Lung Cancer
SF	Scatter factor receptor
TBE	Tris Borate EDTA buffer
TGFα / TGFβ	Transforming Growth Factor-alpha /-beta
TGN	Trans-Golgi Network

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INTRODUCTION

1. CURRENT VIEW OF HUMAN BREAST CANCER

It is well known that tumor suppressor genes and oncogenes are two classes of genes that are critical in the molecular genetics of cancer (Bishop. 1991). Cancer is a process involving not only activation of oncogenes but also loss of tumor suppressor genes. Previous studies found that genetic changes during tumor progression involve the interaction of oncogenes and tumor suppressor genes with growth-regulatory factors. A common link between the actions of growth-promoting steroids and growth factors in diverse tissues is expression of the nuclear oncogenes. Several signal transduction pathways of growth-regulatory stimuli are involved during nuclear oncogenesis. They include the growth factor-induced MAP kinase pathway, the phospholipase C-PCK pathway, and the cytokine-induced JAK-STAT pathways (Roberts. 1992; Silvennoinen, Schindler, Schlessinger, & Levy. 1993; Alkhalaf & Murphy. 1992). The products of three nuclear protooncogenes c-myc, c-fos, and c-jun are found to be induced by both estrogen and progesterone in breast cancer (Dubik & Shiu. 1988; van der Burg, de Groot, Isbrucker, Kruijer, & de Laat. 1991; Alkhalaf & Murphy. 1992). Recent studies also find that tumor suppressor genes such as p53, Rb-1, BRCA-1 are important in familial inherited breast cancer (Dickson & Lippman. 1995). In summary, the function of growth factors, hormones and their relationship with oncogenes and tumor suppressor genes needs to be defined better in order to advance our knowledge in the growth regulation of breast cancer.

(1) Hormonal regulation of proliferation and differentiation in normal breast development

Hormonal factors include the endocrine steroids, peptides, and other molecules produced by the secretory cells of the ovary, pituitary, endocrine pancreas, thyroid, and adrenal cortex. They regulate cellular functions through interactions with nuclear or cell surface receptors. Hormonal factors can be further divided into four types according to their regulatory function. The first are paracrine hormones, synthesized by one cell type and which modulate the same or different cell type. The second are juxtacrine factors which exist on a cell surface and communicate with adjacent cells by contact. The third are autocrine hormones which are released by one cell type and act back on the same cell type through surface or intracellular receptors such as steroid receptors. The fourth are endocrine hormones which are synthesized by many endocrine organs and released through the blood stream to act on target organs (Dickson & Lippman. 1995). A similar hierarchy of growth-regulatory molecules exists in both breast and prostate gland and their cancers (Murphy. 1994). Figure 1 shows the hierarchy of growth-regulatory molecules (Dickson & Lippman. 1995).

The breast originates from the embryonic epithelium. It is different from other exocrine glands which form an entire secretory system long before birth in that the immature mammary gland develops only a duct system and keeps this form till puberty. Normally there are three distinct stages with dramatic structural changes in breast differentiation and proliferation.

A. Prepubertal Breast

The first sign of mammary gland, in the fifth week of human fetal development, is the ectodermal primitive milk streak of thickened epithelium around the ventral middle with extensions from the axial to the groin (Vorherr. 1974). By 12 to 14 weeks, the nipple and areolar are formed by the smooth muscle fibers of the differentiated mesenchymal cells.

The epithelium buds, then branches out into the subcutaneous tissue and forms the ductal system and the secretory alveoli (Hughes. 1950). At 20 weeks, fat and vascularized connective tissue increases and leads to a four-fold increase of mammary gland growth at 32 to 40 weeks. The embryological stage ends with pigment development in the nipple-areolar complex. From the neonatal to the prepubertal period, only slight longitudinal growth and branching of the primary ducts are observed. Studies in rodents have revealed that the sexually dimorphic pattern of mammary growth in rodents are determined by fetal gonadal secretions; in humans this process is less well understood (Imperato McGinley, Binienda, Gedney, & Vaughan, Jr. 1986).

B. Puberty

During puberty, the female breast is rapidly enlarged with ductal elongation and branching due to estrogen and progesterone, the ovarian hormones which are regulated by pituitary hormones (Monaghan, Perusinghe, Cowen, & Gusterson. 1990). Parenchyma and stroma are the two major elements of mature female breast tissue. The parenchymal structure is just like a tree with extensive branched ducts surrounded by collagen fibers which converge to the nipple. The relatively large lactiferous sinus is the main component of the ductal system which functions as 22 collecting duct along the main conducting lactiferous duct (or inter

lobular ducts) to the nipple. Each terminal intralobular duct drains a lobe and consists of 20 to 40 lobules made up of grape-like clusters of 10 to 100 alveoli (Parks. 1959). Stroma is made up of adipose and connective tissue. During the menstrual cycle, ductal growth is under positive regulation by ovarian estrogen, whereas proliferation and regression of secretory acini, budding from the terminals of introlobular ducts, are due to the cyclic levels of ovarian progesterone (Longacre & Bartow. 1986; Fanger, Ree. 1974).

C. Pregnancy-lactation

This is the final stage in differentiation of lobuloalveolar development and it is dependent on many endocrine hormones: prolactin, growth hormone (and its local mediator IGF-I), insulin, glucocorticoids, estrogen and progesterone (Ruan, Newman, & Kleinberg. 1992). When lobuloalveolar growth is completed the gland is ready for lactation. During the last trimester of pregnancy and post- lactation, both colostrum (the first milk secretded by a mammal after parturition) and milk are known to be rich sources of polypeptide growth factors and growth factor-binding proteins. These may be important not only in the regulation of growth and secretory functions of mammary gland in the mother, but also in neonatal immunocompetence, endocrine function, and metabolism (Grosvenor, Picciano, & Baumrucker. 1993). Once breast-feeding has stopped, programmed death (apoptosis) occurs in differentiated luminal cells with withdrawal of the steroid hormones, growth factors and other hormones of pregnancy (Strange, Li, Saurer, Burkhardt, & Friis. 1992).

(2) Hormonal and growth factors control of breast cancer

Breast cancer affects 1 in 9 women in North America in the last twenty years. Established risk factors in breast cancer include family history, a prolonged reproductive period, late pregnancy, excessive consumption of alcohol, high fat diet and cigarette smoking (Dickson & Lippman. 1995). Breast cancer is primarily a postmenopausal disease with possible mechanisms involving prolonged exposure of the breast to ovarian hormones, DNA damage, deregulated growth factors synthesis, oncogenic activation, and deregulated cell cycle-associated genes in the breast (Dickson & Lippman. 1995; Dickson & Lippman. 1992).

A. Hormonal control of breast cancer

Recent studies have shown that both ovarian estrogen and progesterone are necessary to support initial tumor formation and early tumor growth in rodent models of carcinogen-induced and spontaneous mammary cancer (Robinson & Jordan. 1987). It is believed that in both the malignant rodent and human breast, estrogen, acting through its receptors (ER), induces expression of the progesterone receptor (PR). Both hormones, interacting concordantly, then moderate other genes, such as growth factors, oncogenes and cell cycle-associated genes (Clarke & Sutherland. 1990).

In all tissue, a single ER subunit is encoded by a single gene; the complete ER is formed through homodimerization and complex formation with additional proteins, such as heat shock proteins identified in breast cancer cells and also other cells (Smith & Toft. 1993). PR in mammary tissue is formed by both homo-and heterodimerization of three different PR subunits encoded by a single gene (Wei & Miner. 1994). The heterogeneity of PR partners in breast cancer seems to permit a significant variation in dimerization patterns and results in recognizing specific ligands with respect to agonist vs. antagonist (Tung, Mohamed, Hoeffler,

Takimoto, & Horwitz. 1993). Because of the important functions of ER and PR, measurements of their levels are currently used to predict prognosis of patients with breast cancer and as an indication of benefit from antihormonal therapy. But the results are not absolutely precise; more than 60 % of human breast cancer are ER positive, but only two-thirds of these ER-positive tumors are expected to respond to endocrine therapy (Osborne, Yochmowitz. 1980). Additionally, 5-10 % of the patients designated ER-negative respond to endocrine therapy (Edwards, Chamness. 1979). A previous study also found that only 70 % of PR-positive and 25-30 % of PR-negative tumors respond to hormone therapy (Dickson & Lippman. 1995). PR expression is known to be positively regulated by estrogen in both cancer cell lines and normal endometrial tissue (Eckert, Katzenellenbogen. 1981). One current active area of research is to study whether the alternatively spliced or mutant ERs are activated in breast cancer (Pfeffer, Fecarotta, Castagnetta, & Vidali. 1993).

Other research focuses on how the cellular enzyme PKC acts in down-modulation of ER mRNA and function. It has been shown that stimulation of ER-positive breast cancer cells with an activator of PKC leads to down-regulation of ER coincident with loss of its mRNA, and to phosphorylation of residual ER coincident with loss of its function. PKC is expressed to an elevated extent in ER-negative and drug-resistant breast cancer relative to ER positive breast cancer (Saceda, Knabbe, Dickson, et al. 1991). Other studies suggest that several factors such as IGF-I, cAMP, dopamine agonists, and other hormones not only phosphorylate ER and PR but also phosphorylate steroid receptors (Aronica & Katzenellenbogen. 1993). In addition, by forming heterodimers or by modulating chromatin interactions of ER / PR, ER / PR function may be regulated by receptors for other steroids (9 retinoids, vitamin D) (Segars, Marks, Hirschfeld, et al. 1993).

B. Growth factors and their receptors in tumor growth and progression

One active research area is the involvement of growth regulatory molecules (oncogenes or protooncogenes) and locally acting polypeptide hormones (growth factors) in steroid action. Four members of the Epidermal growth factor receptor (EGFR) family and all of the EGF-related growth factors have been detected in breast cancer (Dickson & Lippman. 1995). The EGF growth factor family includes: TGF α; EGF and amphiregulin (a heparin-binding factor) (Dickson & Lippman. 1995). The EGFR-related receptors include c-erbB$_2$, c-erbB$_3$, erbB$_4$ (Higashiyama, Abraham, Miller, Fiddes, & Klagsbrun. 1991; Plowman, Green, Culouscou, Carlton, Rothwell, & Buckley. 1993; Plowman, Culouscou, Whitney, et al. 1993). Additionally at least five other families of growth factors such as insulin-like growth factors (IGF-I and IGF-II), platelet-derived growth factors (PDGF) A and B, and fibroblast growth factors (FGF) are found to be expressed in breast cancer (Goustin, Leof, Shipley, & Moses. 1986; Heldin & Westermark. 1984; Rao, Steck, Mohanam, Stetler Stevenson, Liotta, & Sawaya. 1993). A more recent study also found that vascular endothelial growth factor (VEGF), a tyrosine kinase receptor-binding factor of a separate class, is produced in breast cancer (Ferrara, Houck, Jakeman, & Leung. 1992). Mammary-derived growth factor 1 (MDGF-1) recently has been found in both human milk and conditioned medium from human breast cancer cell lines (Bano, Lupu, Kidwell, Lippman, & Dickson. 1992).

TGFα family of growth factors

TGFα has the same biological effects as EGF in both cultured human and mouse mammary epithelial cell lines and this may due to its structural similarity to EGF (Salomon, Perroteau, Kidwell, Tam, & Derynck. 1987). By *In situ* hybridization studies in both rodent

and human breast tissue, TGFα mRNA has been detected during the proliferative, lobuloalveolar development stage in mammary epithelium (Liscia, Merlo, Ciardiello, et al. 1990). Further immunohistochemical studies in the mouse gland found that TGFα expression occurs in the basal epithelial, and bud cap cell, while EGF expression is in scattered ductal luminal secretory cells (Snedeker, Brown, & DiAugustine. 1991). *In vitro* studies also found that TGFα mRNA, amphiregulin protein, and EGFR are expressed in proliferating human mammary epithelial cells (Bates, Valverius, Ennis, et al. 1990). Here TGFα and amphiregulin act as autocrine growth factors in either normal or immortalized human mammary epithelial cells in mass culture, and their proliferation function can be reversibly inhibited by an anti-EGFR body or heparin (Bates, Valverius, Ennis, et al. 1990).

Gene transfection studies in human breast cancer cell lines and rodent fibroblasts cell lines have generated several different results. Overexpression of TGFα in an immortal but nontumorigenic mouse mammary epithelial cell line, NOG-8, only induces anchorage-independent growth, but not full tumorigenicity (Shankar, Ciardiello, Kim, et al. 1989). Overexpression of TGFα in fully malignant breast cancer MCF-7 cells, which have low levels of EGFR, either *in vitro* or *in vivo* in nude mice gives no significant growth advantage to the cell (Clarke, Brunner, Katz, et al. 1989). In contrast, when TGFα was overexpressed in immortalized rodent fibroblasts, a transformation to full tumorigenicity was achieved (Rosenthal, Lindquist, Bringman, Goeddel, & Derynck. 1986). One possible explanation is that the level of secretion of TGFα in breast cancer and rodent fibroblasts is correlated with expression of other protooncogenes. A direct association between TGFα production, c-ras^{H} protooncogene expression, and malignant transformation has been confirmed *in vitro* (Ciardiello, McGeady, Kim, et al. 1990). There are still many unknown details about the

oncogene-like function of TGFα and other family members and their relationships to the expression of protooncogenes and oncogenes (Dickson & Lippman. 1995).

Transgenic mouse studies with TGFα overexpression (with MMTV or metallothionine promoters) in the mammary gland have found hyperproliferative glands induced by TGFα, often resulting in mammary cancer after multiple pregnancies (Coffey, Jr., Meise, Matsui, Hogan, Dempsey, & Halter. 1994).

TGFβ family of growth factors

TGFβ is found in normal and malignant mammary epithelium and in human milk (McCune, Mullin, Flanders, Jaffurs, Mullen, & Sporn. 1992). TGFβ has three isoforms and once produced, they are only found in the stromal matrix-surrounding ducts and not in the matrix of growing end buds and lateral branches (Mieth, Boehmer, Ball, Groner, & Grosse. 1990). During midpregnancy and lactation, TGFβ production is decreased and TGFβ accumulates in the stroma around alveoli. This suggests that TGFβ may function to suppress lactation (Jhappan, Geiser, Kordon, et al. 1993). An enhanced production of TGFβ in breast cancer with its accumulation in the stromal matrix may be important in tumor angiogenesis (Stampfer, Yaswen, Alhadeff, & Hosoda. 1993). Overexpression of TGFβ also may indicate an aberrant tumor-host interaction in breast cancer, although TGFβ serves as a growth-inhibitory factor in the normal gland (Travers, Barrett Lee, Berger, et al. 1988). *In vitro* studies in some cell lines also suggest that TGFβ may induce cell invasion of an artificial basement membrane barrier (Welch, Fabra, & Nakajima. 1990). Some transgenic mouse studies with TGFβ targeted to the pregnant mammary epithelium with the MMTV promoter

have found that TGFβ can inhibit both alveolar development and lactation (Jhappan, Geiser, Kordon, et al. 1993).

Recent studies have shown that TGFα, amphiregulin, and $TGF\beta_2$ are under transcription control by estrogen and antiestrogen, but little is known about effects of progestins and antiprogestins (Saeki, Cristiano, Lynch, et al. 1991; Martinez Lacaci & Dickson. 1996). *In vitro* studies confirm that in special cell culture conditions in plastic dishes, estrogen can stimulate proliferation of hormone-responsive breast cancer cell lines while progestins have opposite effects. On the other hand in anchorage-independent colony formation in soft agar culture, both estrogen and progesterone have the same growth stimulatory function. Under the above cell culture conditions, estrogen- and progesterone-induced TGFα plays at least a regulatory role in steroid control of growth (Reddy, Yee, Hilsenbeck, Coffey, & Osborne. 1994; Kenney, Saeki, Gottardis, et al. 1993).

Insulin-like growth factors

Studies of insulin - like growth factors (IGFs) have confirmed that IGF-II, is stimulated by estrogen in some hormone-dependent breast cancer cell lines *in vitro*, while antiestrogens have an inhibitory effect. Estrogens and antiestrogens can also regulate cellular responsiveness through altered expression of type 1 IGF receptor, IGF-binding protein-3 (both of which are estrogen induced), and IGF binding protein-4 (estrogen inhibited) (Pratt & Pollak. 1993; Stewart, Johnson, May, & Westley. 1990; Huynh, Tetenes, Wallace, & Pollak. 1993).

Gene transfection studies have found that with overexpression of either IGF-II or $erbB_2$ little growth-enhancement was noted. But upon transfection of FGF-4 or FGF-1 into MCF-7 cells, strong enhancement of tumor growth and metastasis occurs(McLeskey,

Kurebayashi, Honig, et al. 1993).

In vivo studies in nude mice have shown additional characteristic roles of growth factors in breast cancer proliferation. When nude mice are infused with TGFβ_1 in order to block proliferation of the MDA-MB-231 breast cancer cell line, the tumor growth was unaffected *in vivo*, but the animals exhibited cachexia, multiple organ fibrosis, and spleen regression (Zugmaier, Paik, Wilding, et al. 1991).

Thus the functions of growth factors can be divided into two groups dependent on their different effects on cell proliferation, with TGFα, IGF and family members stimulating proliferation, and TGFβ, heregulins (TGFα-related ligand subfamily), and other inhibitory factors inhibiting proliferation. In cancer an aberrant effect of growth factors may be due to growth factor overexpression, perturbation of the signal transduction mechanism, or loss of tissue compartmentalization. In addition, these above actions may be important in tumor growth and metastasis (TGFβ, FGF), desmoplasia and collagen deposition (MDGF-1, TGFα, TGFβ), and immune suppression (TGFβ) (Dickson & Lippman. 1995).

C. EGF Receptor (EGFR) Family

Clinical studies show that the absence of estrogen receptor (ER) is often correlated with high expression of EGFR in both hormone-dependent and hormone-independent breast cancer cell lines and primary tumors (Klijn, Berns, Schmitz, & Foekens. 1992). This observation suggests a mechanistic link between up-regulation of EGFR and hormone independence (Fitzpatrick, Brightwell, Wittliff, Barrows, & Schultz. 1984; Klijn, Berns, Schmitz, & Foekens. 1992). Similar results were obtained with studies using a panel of breast cancer cell lines, with EGFR expression levels varying by more than 2-fold when ER+ and ER- cells are compared (Davidson, Gelmann, Lippman, & Dickson. 1987). This observation

may be due to a transcription-enhancing element, which is located in the first intron of the EGFR gene and is selectively stimulated in ER negative breast cancer cells (Segatto, Lonardo, Wexler, et al. 1991). An opposite affect of ER and EGFR can be observed when hormones and drugs such as estrogen and phorbol esters are used to alter the expression of the two receptors (Hudson, Santon, & Gill. 1989; Lingham, Stancel, & Loose Mitchell. 1988; Mukku & Stancel. 1985; Saceda, Lippman, Chambon, et al. 1988).

The EGFR is a 170 kDa transmembrane glycoprotein with an intrinsic tyrosine kinase activity. Intracellular internalization and down-regulation of the receptor occurs when EGF binds to EGFR, and the binding complex further leads to kinase activation, autophosphorylation of EGFR, and phosphorylation of other substrates (Cohen. 1982; Hunter, Cooper. 1981).

One function of EGFR may be to modulate the proliferation of tissues during fetal development. In the adult, EGFR may act to modulate tissue proliferation during pregnancy (Fisher & Lakshmanan. 1990). All members of the EGFR have a three characteristic disulfide linkages in the secondary structure, which may contribute to the three-dimensional structure of the receptors and to growth factor action (Todaro, Rose, Spooner, Shoyab, & Plowman. 1990). Polypeptide factors that bind to the EGFR are synthesized from transmembrane precursors that are cleaved by proteolytic enzymes to yield fully active form. Several of the receptor kinase substrates are involved in signal transduction. Those reported include: phospholipase C, phospholipase D, phosphoinositol-3 kinase, GRB_2/ SOS, RAS/ GAP, RAF kinase, mitogen activated kinase (MAP) kinase, the ribosomal S-6 kinase (Muthuswamy, Siegel, Dankort, Webster, & Muller. 1994; Roberts. 1992).

The MAP (mitrogen activated kinase) kinase family is an important mitogenic

phosphorylation signal. It is activated by diverse phosphotyrosine, phosphoserine, phosphothreonine kinase pathways, and some G protein-mediated pathways. MAP kinase is also involves in a coordinated series of events leading to mitosis (Nishida & Gotoh. 1993).MAP kinase phosphorylates at least three nuclear protooncogenes c-myc, c-jun, and c-ets and it is one regulator of their transcriptional activity (Franklin, Unlap, Adler, & Kraft. 1993; Kato & Dang. 1992). Receptors with tyrosine kinase activity such as EGFR and c-erbB$_2$ (an EGFR-related protein) are known to regulate growth via a complex signal transduction cascade involving sequential phosphorylations and activation of the RAS and RAF oncogene products, MAP kinase kinase, MAP kinase, and induction of transcription-regulatory factors (Sadowski, Shuai, Darnell, Jr., & Gilman. 1993). Recent studies have shown that cAMP can inhibit RAS activation of RAF through inhibition of phosphorylation, thus producing a natural inhibitory pathway in the tyrosine kinase signal transduction cascade and blocking proliferative stimuli (Cook & McCormick. 1993; Wu, Dent, Jelinek, Wolfman, Weber, & Sturgill. 1993). Some of the most common genetic alterations in breast cancer involve the amplification of the c-erbB$_2$ receptor gene, the c-myc gene, and the genes encoding cyclins D and E (Hunter & Pines. 1991; Lewin. 1991).

Previous studies have shown that about 10-30 % of breast, stomach, and ovarian cancers overexpress the c-erbB$_2$ gene product; similar results have been reported in cancers from other organs including adenocarcinomas of the lung (Kern, Schwartz, Nordberg, et al. 1990),pancreas (Hall, Hughes, Staddon, Richman, Gullick, & Lemoine. 1990), and endometrium (Berchuck, Rodriguez, Kinney, et al. 1991). A recent transgenic mouse study has shown that c-erbB$_2$ gene expression stimulates metastatic breast tumors (Guy, Schaller. 1992). The co-expression of the c-src protooncogene product with the c-erbB$_2$ gene product

in this transgenic model appears to be critical in the tumorigenic pathway (Muthuswamy, Siegel, Dankort, Webster, & Muller. 1994). c-erbB$_2$ expression has been correlated with poor prognosis in cancer patients (Gusterson, Gelber, Goldhirsch, et al. 1992; Wright, Cairns, Cantwell, et al. 1992; Muss, Thor, Berry, et al. 1994). Current research now focuses on the potential contribution of c-erbB$_2$ to tumor metastasis and drug resistance.

D. Oncogenes And Tumor Suppressor Genes

A number of nuclear oncogenes play important roles in breast cancer. In ER positive and hormone dependent breast cancer cell lines, c-myc is directly regulated by estrogen (Dubik & Shiu. 1992; Dubik & Shiu. 1988). The c-myc oncoprotein is also necessary for estrogen induction of proliferation of breast cancer (Watson, Pon, & Shiu. 1991). The c-myc protooncogene may be important in breast cancers of older women, as c-myc is likely to be amplified, especially in postmenopausal women (Escot, Theillet, Lidereau, et al. 1986). In addition c-myc and TGF α may play a role in epithelial transformation when they are overexpressed (Lee, Raymond, Tsao, Lee, Earp, & Grisham. 1991; McCune, Mullin, Flanders, Jaffurs, Mullen, & Sporn. 1992).

Tumor suppressor genes such as p53 and Rb-1 are important in breast cancer and mutations in these genes occurs in approximately 40 % and 20 % of breast cancer cases, respectively (Dickson & Lippman. 1995). Recently, two tumor suppressor genes called BRCA-1 and BRCA-2 have been found to be important in the inherited forms of breast and ovarian cancers. BRCA1 gene was first localized to chromosome 17q (Hall, Lee, Newman, et al. 1990) and has been cloned (Miki, Swensen, Shattuck Eidens, et al. 1994). It is composed of 22 coding exons distributed over roughly 100 kb of genomic DNA. Mutations

in BRCA1 have been linked to more than 45 % of sitespecific, inherited breast cancer and 80 % of families with breast and ovarian cancer (Easton, Bishop, Ford, & Crockford. 1993). BRCA1 mutations are not often found in sporadic breast cancers and only about 10 % of sporadic ovarian cancers have been found to have BRCA1 mutations (Futreal, Liu, Shattuck Eidens, et al. 1994; Hosking, Trowsdale, Nicolai, et al. 1995). BRCA2 is composed of 27 exons distributed over roughly 70 kb of genomic DNA (Tavtigian, Simard, Rommens, et al. 1996). Mutations in BRCA2 are frequently found in the hereditary form of human breast cancers(Tavtigian, Simard, Rommens, et al. 1996).

BRCA1 complementary DNA encodes a 1863-amino acid protein whose predicted structure includes two conserved zinc finger domains near the NH_2-terminus and an acidic COOH-terminal domain, suggesting that BRCA1 protein is a transcription factor(Vogelstein & Kinzler. 1994; Miki, Swensen, Shattuck Eidens, et al. 1994). Several lines of evidence suggest that BRCA1 is a tumor suppressor gene mainly functioning in breast, ovarian and prostate cancer. An *in vitro* study has shown that overexpression of the wild-type BRCA1 gene inhibits growth of all breast and ovarian cancer cell lines, but not colon or lung cancer cells or fibroblasts, and mutant BRCA1 showed no effect on growth of breast cancer cells (Holt, Thompson, Szabo, et al. 1996); other studies on elucidating the role of BRCA1 in the regulation of growth and differentiation of the breast have found that BRCA1 was expressed rapidly during mouse embryonic development and in adult mouse tissues, and during postnatal mammary gland development (Marquis, Rajan, Wynshaw Boris, et al. 1995). Further investigation has also found that BRCA1 expression in the mammary gland was stimulated during puberty, pregnancy, and following treatment of ovariectomized animals with 17β-estradiol and progesterone (Marquis, Rajan, Wynshaw Boris, et al. 1995). This results

support the notion that BRCA1 plays an important role in the ovarian hormone responsive regulation of breast growth and differentiation (Marquis, Rajan, Wynshaw Boris, et al. 1995). In addition, epidemiological and genetic studies have implicated BRCA1 as a tumor suppressor gene in prostate cancer (Arason, Barkardottir, & Egilsson. 1993; Ford, Easton, Bishop, Narod, & Goldgar. 1994). An *in vivo* study on nude mice with MCF-7 tumors has shown that overexpression of wild-type BRCA1 inhibited both MCF-7 cells and tumor growth in nude mice resulting in an increased survival (Holt, Thompson, Szabo, et al. 1996). Another studing using transgenic mice has shown that a mutation in one BRCA1 allele can produce abnormal neuroepithelial development resulting in early embryonic lethality (Gowen, Johnson, Latour, Sulik, & Koller. 1996). In order to characterize the regulation and function of the BRCA1 gene, a microscopy-directed method was used to compare mRNA from normal and neoplastic tissues from patients with non-hereditary (sporadic) breast cancer (Jensen, Page, & Holt. 1994). *In situ* hybridization results showed that BRCA1 mRNA levels are dramatically reduced during the transition from carcinoma to invasive cancer. In general, BRCA1 serves as a negative regulator of mammary epithelial cell growth, which is expressed at diminished levels in the invasive forms of sporadic breast cancer (Thompson, Jensen, Obermiller, Page, & Holt. 1995).

The functional relationship between sex steroids, growth factors, oncogenes, and tumor suppressor genes in the cell cycle is shown in Figure 2 (Dickson & Lippman. 1995).

E. Angiogenesis and Metastasis in Breast Cancer

Cancer metastasis is a consequence of an interaction between tumor cells and host factors. Cancer metastasis is multi-step, and is believed to occur by the following processes:

the initial transforming event occurs, tumor cells grow progressively. When the tumor mass becomes excessive, the local nutrient supply through diffusion is not enough, thus a neovascular network from the surrounding host tissue is established. Local invasion of the host stroma by tumor cells occurs through lymphatic circulation and other mechanisms, allowing the tumor cells into the blood circulation. Those cells that survive the circulation must aggregate and arrest in the capillary beds of distant organs where secondary invasion begins.

In breast cancer, the cell-cell adhesion molecule E-cadherin is considered to function like a tumor suppressor gene. Experimental studies have shown that loss of E-cadherin expression is correlated with increased motility, fibroblastic morphology, and increased invasion (Sommers, Thompson, Torri, Kemler, Gelmann, & Byers. 1991). In addition, loss of ER and PR expression in breast cancer is thought to correlate with invasive propensity. These mechanisms may be explained by dysfunctional desmosomal and cytoskeletal proteins (Thompson, Paik, Brunner, et al. 1992; Boyer, Tucker, Valles, Franke, & Thiery. 1989).

Metastases usually implies an ability to break loose, enter the bloodstream or lymphatic vessels, and form secondary tumors. Overexpression of nonintegrin, a 67-kDa receptor for laminin-binding protein, has been reported in progression of breast and colon cancer (Friedrichs, Ruiz, Franke, Gille, Terpe, & Imhof. 1995; Liotta, Steeg, & Stetler Stevenson. 1991). Other studies shows that the heterodimeric integrins are important in cancer metastasis (Schwartz & Ingber. 1994). A tyrosine kinase called FAK (focal adhesion kinase) may be involved in cell adhesion and is up-regulated in invasive breast cancer (Weiner, Liu, Craven, & Cance. 1993).

Previous studies have found that several local proteolytic enzymes play an important

role in cancer metastasis. They include a 92 kDa matrix metalloprotease-9 (MMP-9), collagen IV-selective degrading enzymes, urokinase (the tumor cell-secreted plasminogen activator), cathepsin D, an 80-kDa broad substratum matrix-degrading metalloproteinase, cathepsin B, and cathepsin L (Castiglioni, Merino, Elsner, Lah, Sloane, & Emmert Buck. 1994; Seidah, Day, Hamelin, Gaspar, Collard, & Chretien. 1992; Rao, Steck, Mohanam, Stetler Stevenson, Liotta, & Sawaya. 1993; Shi, Torri, Yieh, Wellstein, Lippman, & Dickson. 1993). Current reports show that MMP-9 and type IV collagenase mediate metastatic activity in human breast cancer cell lines (Liu, Connolly, & Rose. 1996), but that cathepsins B, D, and L seem to have no influence in human breast cancer cell lines (Castiglioni, Merino, Elsner, Lah, Sloane, & Emmert Buck. 1994). It is believed that local invasion of the cancer across the basement membrane to the stromal area is the first step of metastasis. Local proteolysis and tumor cell motility are thought to contribute to this process (Dickson & Lippman. 1995).

Matrix metalloproteases (MMPs) are believed to be important local proteolytic enzymes involved in metastases. Normally MMPs are active in tissue remodeling, repair and destruction (Woessner, Jr. 1991; Matrisian. 1992). The enzymes can hydrolyze proteins of the extracellular matrix, precursor proteins, proteinase inhibitors and growth factor-binding proteins. Human MMPs include interstitial collagenase, neutrophil collagenase, collagenase-3, gelatinases A and B, stromelysin-1, stromelysin-2, stromelysin-3, matrilysin, and metalloelastase (Will & Hinzmann. 1995). The activity of MMPs requires Zn^{2+} and Ca^{2+} and inhibited by tissue specific inhibitors (Woessner, Jr. 1991; Matrisian. 1992). Normally synthesis, activation and inhibition of MMPs are strictly controlled. Abnormal changes of the above balance are found to be involved in tumor cell invasion (Stetler Stevenson, Liotta, & Kleiner, Jr. 1993).

Urokinase and its inhibitor PAI-I (plasminogen activator inhibitor-1) are secreted by stromal cells adjacent to invasive breast cancer; they are both believed to be an important indicators of poor prognosis in breast cancer (Crowley, Cohen, Lucas, Liu, Shuman, & Levinson. 1993; Stack, Gray, & Pizzo. 1993).

Recent research suggests that several growth factors also involved in cancer metastasis. These factors include the FGF family members (Jouanneau, Gavrilovic, Caruelle, et al. 1991), hepatocyte growth factor (HGF), IGF-II and IGF-I. It has been suggested that IGF-II is expressed by breast tumor-derived fibroblasts, and IGF-I is expressed by normal breast fibroblasts (Cullen, Lippman, Chow, Hill, Rosen, & Zwiebel. 1992; Sonnenberg, Meyer, Weidner, & Birchmeier. 1993).

It is also believed that neovascularization (blood vessel invasion into the tumor area) is a key step in cancer metastasis (Fidler. 1990). Several growth factors,such as the FGFs, EGF-related factors, TGF-βs, HGF, and vascular endothelial growth factor (VEGF) are considered to be involved in angiogenesis (Folkman & Shing. 1992; Millauer, Wizigmann Voos, Schnurch, et al. 1993). According to some *in vivo* models of human breast cancer metastasis, hormone-independent breast cancer cells are more likely to be invasive in the nude mouse than hormone-dependent cells (Thompson, Paik, Brunner, et al. 1992). One of the hormone-independent cell lines, MDA-MB-435, has been developed into a hematogeneic metastasis model in the nude mouse. This cell line can produce micrometastases in 6-9 months at the inoculation site (Price, Polyzos, Zhang, & Daniels. 1990; Meschter, Connolly, & Rose. 1992; Noguchi, Ohta, Kitagawa, et al. 1992).

2. Proprotein Convertases

It is well known that many eukaryotic proteins such as hormones, neuropeptides,

cellular secreted proteins, growth factors, and their membrane receptors do not have biological activity unless post-translation modulation occurs. One group of enzymes responsible for post-translational processing are called proprotein convertases. Mammalian proprotein convertases perform endoproteolytic cleavage of proproteins on the C-terminal side of dibasic amino acid motifs of several types: type I site cleaves the motif Arg-X-Lys / Arg-Arg (R-X-K / R-R), type IIA site cleaves the motif X-X-Arg / Lys-Arg (X-X-K / R-R), and type IIB site cleaves the motif Lys / Arg-X-X-Arg (K / R-X-X-R) (Mbikay, Seidah, & Chretien. 1993). The first eukaryotic proprotein-processing enzyme found was the Kex2 protease of the yeast *Saccharomyces cerevisiae*. The yeast Kex2 protein and mammalian proprotein convertases contain a bacterial subtilisin-like catalytic domain (Seidah, Day, & Chretien. 1993; Seidah, Day, Marcinkiewicz, & Chretien. 1993; Fuller, Brake, & Thorner. 1989; Fuller, Brake, & Thorner. 1989). The mating of yeast haploid cell types a and α to form a / α diploids in the yeast *Saccharomyces cerevisive* requires Kex2 to cleave pro-α-factor at four Lysine- Arginine pairs of basic residues. A specific carboxypeptidase, Kex1, cleaves pro-α-factor at the C-terminal. Another enzyme involved in this process known as dipeptidyl aminopeptidase A, further cleaves the protein to a secretable form. Previous studies have shown that Kex2 is a Ca^{2+}-dependent serine proteinase belonging to the subtilisin protein family. Kex2 exhibits a marked selectivity of cleavage C-terminal to Lys-Arg, Arg-Arg, and pro-Arg- sequences. Also Kex2 is involved in processing of α-mating factor and killer toxin precursors at dibasic sites (Fuller, Sterne, & Thorner. 1988; Julius, Brake, Blair, Kunisawa, & Thorner. 1984).

To date, seven mammalian proprotein convertases members have been reported. A summary of the structures of these mammalian proprotein convertases is shown in Figure 3.

(1) Furin

Furin, the first human gene product homologous to Kex2 was discovered through computer alignment of the amino acid sequences surrounding the active sites Ser, His, and Asn (Bryan, Pantoliano, Quill, Hsiao, & Poulos. 1986). The human furin gene lies upstream of the tyrosine kinase *fps / fes* oncogene (Roebroek, Schalken, Leunissen, Onnekink, Bloemers, & Van de Ven. 1986). Furin is to date, the best characterized enzyme of the mammalian subtilisin-kexin like proprotein convertases. Northern blot analysis has shown that a 4.4 kb furin transcript is ubiquitously distributed in mammary tissues and cell lines.(Seidah, Chretien, & Day. 1994). Furin, like the yeast kexin, is the only member of the mammalian proprotein convertases that has a transmembrane domain (Fuller, Brake, & Thorner. 1989). Thus the subcellular location of furin only exists between the *trans*-Golgi network (TGN) and cell membrane (Vey, Schafer, Berghofer, Klenk, & Garten. 1994; Molloy, Thomas, VanSlyke, Stenberg, & Thomas. 1994). Two signals found in the cytoplasmic domain of the enzyme play a role in its sub-cellular translocation (Schafer, Stroh, Berghofer, et al. 1995). The first is the CPSDEEDEG sequence (residues 771-780), which contains a casein kinase II phosphorylation motif (S / T-D / E-D / E-D / E); this may function to phosphorylate the Ser^{773} and Ser^{775} residues (Bosshart, Humphrey, Deignan, et al. 1994). The phosphorylation / dephosphorylation changes may be correlated with the TGN. The second is a YKGL sequence (residues 757-760). This sequence acts in routeing furin to the endosomes (Schafer, Stroh, Berghofer, et al. 1995). The actual physiological function of the above subcellular translocation is unknown except that furin is believed to be involved in the processing of proteins secreted via the constitutive secretion pathway (Seidah, Day, & Chretien. 1993; Seidah, Day, Marcinkiewicz, & Chretien. 1993).

The human furin gene, located at chromosome 15q25-q26 (Seidah, Chretien, & Day. 1994) was cloned in 1990 from a human liver cell (HepG2) cDNA library (Wise, Barr, Wong, Kiefer, Brake, & Kaufman. 1990). According to the human furin cDNA sequence, a 794 amino acids translation product is predicted. The characteristics of the various domains of this Ca^{2+}-dependent protease includes: (i) a signal peptide related to the secretory pathway; (ii) a pro-region; (iii) a catalytic domain similar to subtilisin-like enzymes; (iv) a P-domain necessary in enzymatic activity with a cysteine-rich region; (v) a hydrophobic transmembrane spanning domain; (vi) a cytoplasmic tail, involved in subcellular translocation (Jean, Boudreault, Basak, Seidah, & Lazure. 1995).

When recombinant vaccinia virus expression system was used to introduce human furin cDNA into BSC-40 cells (African green monkey kidney cell line), a doublet of proteins with the apparent sizes of 90 and 96 kDa, was detected. Only the 96 kDa form undergoes an intramolecular autocatalytic activation by removal of the pro-region of furin in the endoplasmic reticulum (Bresnahan, Leduc, Thomas, et al. 1990; Leduc, Molloy, Thorne, & Thomas. 1992; Vey, Schafer, Berghofer, Klenk, & Garten. 1994). It is interesting to find that furin can cleave more than 25 proteins when co-expressed with substrate precursors *in vivo* (Jean, Boudreault, Basak, Seidah, & Lazure. 1995).(Table I).

Recent studies have shown that the *FUR* gene has three distinct promoters (P1, P1A and P1B). This may help to explain why multiple mRNA isoforms exist ranging in size from 4.0,4.5, 6.8 and 8.4 Kb (Hayflick, Wolfgang, Forte, & Thomas. 1992). However, the 4.4 Kb furin transcript is the major form detectable in most tissues. When synovial cells and NIH-3T3 cells are treated with TGF-β, a significant increase in furin transcript levels are observed. Furin is the major enzyme involving in TGF-β precursor processing (Blanchette, Day.R.

1995).

Several inhibitors of furin have been used to study the post-translational process. One *in vivo* study showed the inhibition of furin-derived processing of HIV-1 gp (glycoprotein) 160 to the gp120 protein, by chloromethylketone (Hallenberger, Bosch, Angliker, Shaw, Klenk, & Garten. 1992). Another similar experiment found that decanoyl-Val-Lys Arg-chloromethylketone can inhibit furin processing of proendothelin-1 *in vivo* (Denault, Claing, D'Orleans Juste, et al. 1995). In addition, α_1-antitrypsin Portland (AT-PDX), a protein-based serine protease inhibitor has been used to abolish furin activity (Lu, Zhang, Molloy, et al. 1993). AT-PDX is now known to be a potent inhibitor of all the members of the proprotein convertases.

(2) PC1

Based on the belief that the sequence around the active sites of serine proteinases is conserved, polymerase chain reaction (PCR) was used to search for Kex2 homologues. This led to the discovery of a second prohormone convertase, PC1 (Seidah, Gaspar, Mion, Marcinkiewicz, Mbikay, & Chretien. 1990; Smeekens, Avruch, LaMendola, Chan, & Steiner. 1991). *In situ* hybridization studies confirmed that PC1 was only found in cells of endocrine and neuroendocrine origin, including the islets of Langerhans, pituitary, adrenal medulla, and brain (Seidah, Marcinkiewicz, Benjannet, et al. 1991; Seidah, Gaspar, Mion, Marcinkiewicz, Mbikay, & Chretien. 1990). PC1 is responsible for cleaving pro-proteins secreted in the regulated secretory pathway (Seidah, Day, & Chretien. 1993; Seidah, Day, Marcinkiewicz, & Chretien. 1993). Mouse PC1 and human PC1 share about 87 % amino acid sequence similarity. The complete cDNA sequence of mPC1, consists of 2516 nucleotides, with a 2259

bp open reading frame. The processed protein consists of 726 amino acids, with three potential N-glycosylation sites. If each glycosylation chain contributes approximate 2000 daltons, the predict molecular weight of mPC1 is about 87 kDa (Seidah, Marcinkiewicz, Benjannet, et al. 1991). Northern blot analysis of AtT-20 cells shows two mPC1-hybridizing bands (3.0 and 4.8 kb) (Seidah, Gaspar, Mion, Marcinkiewicz, Mbikay, & Chretien. 1990). The mPC1 is located on mouse chromosome 13 (Seidah, Marcinkiewicz, Benjannet, et al. 1991).

A substantial body of work have been done in order to understand further the biosynthesis and biochemical properties of PC1. One interesting result demonstrates that when recombinant PC1 was overexpressed in Chinese hamster ovary cells (CHO), PC1 is first synthesized in a precursor form, then quickly undergoes an amino-terminal cleavage event at a very early stage of biosynthesis to generate an 87kDa form. Another study shows that the 87 kDa PC1 protein is calcium-dependent and has enzymatic activity between pH 5.0 and 6.5. The 87 kDa PC1 can be further converted to 74 kDa and 66 kDa forms by carboxyl-terminal cleavages. Compared to the 87 kDa PC1 form, the 74 / 66 kDa PC1 are more active but less stable, and seemed more sensitive to certain protease inhibitors than 87 kDa PC1. These forms have more narrow pH optima (between 5.0 and 5.5) and are activated by higher concentrations of calcium. The above results suggest that an auto-catalysis mechanism may be involved in carboxyl-terminal cleavages of PC1, and may play an important role in proprotein processing (Zhou & Lindberg. 1994; Zhou & Lindberg. 1993).

Another report focused on how the biosynthetic processing pathway of endogenous PC1 in AtT-20 cells (ACTH-producing mouse pituitary tumor cell line) was post-

translationally regulated. The first PC1 translational product is a 94 kDa protein that is quickly converted to an 84 kDa form. The 84 kDa protein is then converted to an endoglycosidase H-resistant form of the 97 kDa protein, and finally to the 74 kDa and 66 kDa species. The post-translational processing of PC1 occurs in two steps: (i) the 94 kDa precursor is quickly converted to the 84 kDa protein by a cleavage of the amino-terminus prosegment; this process may occur in the endoplasmic reticulum; (ii) the 87 kDa protein is converted to the 74 kDa and the 66 kDa protein; this process may take place in cellular compartments such as the *trans*-Golgi network or secretory granules and involve sequential cleavages at the carboxyterminus. These data first show the subcellular translocation of PC1 during proprotein processing (Vindrola, Mayer, Citera, Spitzer, & Espinoza. 1994).

The cleavage specificity of the PC1 has been analyzed by co-expression with a number of prohormones, such as POMC (Benjannet, Rondeau, Day, Chretien, & Seidah. 1991; Thorne, Viveros, & Thomas. 1991), prorenin (Nakayama, Watanabe, Nakagawa, et al. 1992), proenkephalin (Konoshita, Gasc, Villard, et al. 1994), prodynorphin (Dupuy, Lindberg, Zhou, et al. 1994), prorelaxin (Marriott, Gillece Castro, & Gorman. 1992), pro-neurotensin (Rovere, Barbero, & Kitabgi. 1996), secretogranin II to secretoneurin (Hoflehner, Eder, Laslop, Seidah, Fischer Colbrie, & Winkler. 1995), and rabbit pro-LPH (Keller, Zecca, Boukamel, Zwicker, Gloor, & Semenza. 1995), and proneuropeptide Y (Paquet, Massie, & Mains. 1996). In addition, studies on pancreatic hormones suggest PC1 is involved in the processing of insulin (Smeekens, Montag, Thomas, et al. 1992), proglucagon-derived peptides (Rothenberg, Eilertson, Klein, Mackin, & Noe. 1996), and somatostatin (Galanopoulou, Kent, Rabbani, Seidah, & Patel. 1993). In the future, there may be more and more proteins identified as substrates for PC1.

(3) PC2

Just like the discovery of PC1, PC2 was identified and characterized from a human insulinoma using PCR (Seidah, Gaspar, Mion, Marcinkiewicz, Mbikay, & Chretien. 1990; Smeekens & Steiner. 1990). Northern blot analysis revealed the presence of a 2.8 kb and a 5.0 kb hybridizing band in mRNA from insulinomas (Smeekens & Steiner. 1990). The gene was located on human chromosomes 20p11.1-11.2 (Seidah, Marcinkiewicz, Benjannet, et al. 1991). The 638 amino acid sequence of PC2 begins with a 25 amino acid signal peptide. PC2 is synthesized as a 75 kDa propolypeptide which is auto-cleaved to a 68 kDa mature enzyme. It is also believed that PC2 is involved in a regulatory secretory pathway. Further studies have shown that PC2 like PC1, exists in endocrine tissue (Smeekens & Steiner. 1990). Recent reports found that 7B2, a neuroendocrine chaperone protein belonging to the granin family (Huttner, Gerdes, & Rosa. 1991), is intimately involved in proPC2 maturation (Leiser & Sherwood. 1989; Kelly. 1985). In addition, it was found that during intracellular translocation of PC2, pro7B2 can bind both proPC2 and PC2 (Braks & Martens. 1994). PC2 is involved in the precursor processing of: pro-POMC (Benjannet, Rondeau, Day, Chretien, & Seidah. 1991), proinsulin (Bailyes, Shennan, Seal, et al. 1992), pro-islet amyloid polypeptide (IAPP) (Badman, Shennan, Jermany, Docherty, & Clark. 1996), proneuropeptide Y (Paquet, Massie, & Mains. 1996), and pro-glucagon (Rothenberg, Eilertson, Klein, Mackin, & Noe. 1996).

(4) PACE4

Human PACE4 is structurally similar to furin (Kiefer, Tucker, Joh, Landsberg, Saltman, & Barr. 1991). Previous studies show that the catalytic domain of PACE4 exhibits a 70 %

identity to that of PC5; in addition, it has a Cysteine-rich domain (Lusson, Vieau, Hamelin, Day, Chretien, & Seidah. 1993). Northern blot analysis shows that only a 4.4 kb form of PACE4 mRNA is expressed (Dong, Marcinkiewicz, Vieau, Chretien, Seidah, & Day. 1995). PACE4 has been demonstrated to cleave pro-von Willebrand factor (Creemers, Kormelink, Roebroek, Nakayama, & Van de Ven. 1993; Rehemtulla, Barr, Rhodes, & Kaufman. 1993). Rat PACE4 has a tissue and cell line distribution unlike human PACE4 and other reported proprotein convertases, with high expression in the anterior pituitary. The prevalence of PACE4 in the anterior pituitary and the striking effect of thyroid status on PACE4 expression suggest a specific role for PACE4 in processing neuroendocrine peptides (Johnson, Darlington, Hand, Bloomquist, & Mains. 1994).

(5) PC4

PC4, located on human chromosome 21, shows a 2.8 kb major and a 1.9 kb minor PC4 transcript . This proprotein convertase has only been found in testicular germ cells (Mbikay, Raffin Sanson, Tadros, Sirois, Seidah, & Chretien. 1994; Seidah, Day, Hamelin, Gaspar, Collard, & Chretien. 1992).

(6) PC5

PC5, located on human chromosome 9, contains a C-terminal Cysteine-rich domain which is similar to furin and PACE4 (Seidah, Chretien, & Day. 1994). It can be detected in many endocrine and nonendocrine tissues, and the PC5 mRNA (3.8 kb) is expressed at high levels in the rat adrenal gland and gut. PC5 transcript is found increased in the ACTH-stimulated adrenocortical Y_1 cell line, suggesting an upregulation by cAMP (Lusson, Vieau, Hamelin, Day, Chretien, & Seidah. 1993; Nakagawa, Hosaka, Torii, Watanabe, Murakami, &

Nakayama. 1993). Previous studies report that PC5, together with PC1 and PC2, may be important in cleaving gastrointestinal peptides to their mature forms. The conversion of prorenin to renin is a good example (Nakagawa, Hosaka, Torii, Watanabe, Murakami, & Nakayama. 1993). It is recently been confirmed that PC5 plays an important role in processing protein tyrosine phosphatase μ in human umbilical vein endothelial cells (HUVEC) (Badman, Shennan, Jermany, Docherty, & Clark. 1996).

(7) PC7

The latest member of the proprotein convertase family is LPC (PC7) which has been found at a human chromosome translocation breakpoint t (11; 14) (q 23; q 32) is associated with a high grade lymphoma. Northern blot analysis has shown a 3.5 kb transcript is present in human tissues, and a similar size is expressed in mouse tissues. A minor mRNA transcript of 4.5 kb occurs in testis and lung (Meerabux, Yaspo, Roebroek, Van de Ven, Lister, & Young. 1996). In addition, a rat PC7 was recently found which showed high expression levels in the colon and lymphoid tissues (Seidah, Hamelin, Mamarbachi, et al. 1996). As yet, little is known about the substrate specificity and specific physiological functions of PC7.

3. Possible Relationship between Proprotein Convertases and Breast Cancer

Proprotein convertases were first discovered in the late nineteen eighties, and to date, seven mammalian proprotein convertases have been identified (PC1 / PC3, PC2, PC4, PC5 / PC6, PC7, PACE4 and furin). The cDNAs for six human proprotein convertases have also been subsequently cloned (PC1, PC2, PC5, PC7, furin and PACE4). Early studies have concentrated on characterization of proprotein convertases molecular structure, tissue

distribution, subcellular translocation, and substrate specificity of proprotein convertase members. Little is known about their physiological functions. To date, no reports have addressed the biological functions of proprotein convertases in breast cancer.

There is considerable evidence that both normal breast tissue and breast tumors are regulated by autocrine and paracrine hormone factors, such as endocrine steroids, peptides synthesized by the secretory cells of the ovaries, pituitary, endocrine pancreas, thyroid, adrenal cortex, and locally acting hormone-like substances produced by normal and malignant mammary tissues (Dickson & Lippman. 1995). Data suggests that abnormal expression levels of autocrine or paracrine growth factors , together with oncogenes, play a major role in human breast cancer progression (Dubik & Shiu. 1988; Dubik, Dembinski, & Shiu. 1987; Musgrove, Lee, & Sutherland. 1991). The major biological function of proprotein convertases is to convert biologically inactive proproteins into their biologically active forms. Several proprotein convertases have been confirmed to be involved in the processing of a variety of precursor growth factors, including nerve growth factor (NGF), IGF-I and IGF-II, TGF-β_1, endothelin-I, EGF, neurotrophin, PDGF, and several growth factor receptors (Mbikay, Seidah, & Chretien. 1993; Jean, Boudreault, Basak, Seidah, & Lazure. 1995). A possible relationship exists between proprotein convertases and breast cancer development (Cheng, Watson, Paterson, Seidah, Chretien, & Shiu. 1997). Overexpression of proprotein convertases may be involved in the neoplastic process of the breast epithelium by virtue of their ability to produce biologically active growth regulatory factors and / or their receptors. It is also possible, that overexpression of proprotein convertases may, through post-translational processing, inactivate biologically active tumor suppressor proteins. Aberrant expression of proprotein convertases may alter the activities of important cellular regulatory proteins that

in turn influence the progression of breast cancer.

A direct linkage between proprotein convertases and cancer is suggested by a new member of observations. PC7, the seventh and newest member of the proprotein convertases family, is located at a chromosome breakpoint in a subset of lymphomas with a chromosomal translocation t (11; 14) (q 23; q 32) (Meerabux, Yaspo, Roebroek, Van de Ven, Lister, & Young. 1996). A study has shown that furin mRNA is expressed at a high level in non-small cell lung carcinoma (NSCLC) but is undetectable in small cell lung carcinoma (SCLC) and normal lung tissues (Schalken, Roebroek, Oomen, et al. 1987). Similarly, an elevated PC1 mRNA level has also been found in anthother group of lung carcinomas different from SCLC and NSCLC, seldom in SCLC, and undetectable in NSCLCs (Creemers, Roebroek, & Van de Ven. 1992).

In our laboratory, a first step towards elucidating the biological role that proprotein convertases play in growth regulation of breast cancer was to compare mRNA expression of furin, PACE4, PC1, PC2, PC5 and PC7 in 30 primary human breast cancer specimens, 6 human breast cancer cell lines, and 10 specimens of histologically normal human breast tissues specimens. The results showed that mRNAs for furin, PACE4, PC1 and PC7 were expressed in all the tumor tissues and cancer cell lines whereas mRNA of PC5 was expressed in only a small percentage (2 / 30) of tumor tissues and not in breast cancer cell lines. PC2 mRNA was not detectable in either tumor tissues or cancer cell lines. Proprotein convertase mRNAs was not detectable in histologically normal human breast tissues. In addition, an *in situ* hybridization study has localized furin mRNA to the breast carcinoma tumor cells; adjacent fibrous stroma and blood vessel elements were negative for furin gene expression (Cheng,

Watson, Paterson, Seidah, Chretien, & Shiu. 1997). A second study also has shown that PC1 protein, as determined by immunohistochemistry, was elevated in breast carcinoma while PC2 protein was undetectable (Scopsi, Gullo, Rilke, Martin, & Steiner. 1995).

Our results showed that several proprotein convertase mRNAs are detectable in 30 primary human breast tumors. However, mRNA expression levels between convertases and between samples are different. One possible explanation for this observation is that different proportions of tumor cells to non tumor elements exist in different tumor specimens. Another interesting observation is that no significant correlation exists between the levels of PC1 or furin gene expression and steroid hormone (estrogen and progesterone) receptor status in human breast tumors. However, a significant correlation exists between PACE4 gene expression and estrogen receptor content. The significance of these observations remain to be studied (Cheng, Watson, Paterson, Seidah, Chretien, & Shiu. 1997).

The recent discovery of two breast cancer susceptibility genes, BRCA1 and BRCA2 may provide a direct link between proprotein convertases expression and breast cancer phenotype. Breast cancer occurs in both hereditary and sporadic forms. The BRCA1 gene has been shown to be mutated in a subset of kindreds with the hereditary form of breast cancer (Castilla, Couch, Erdos, et al. 1994; Miki, Swensen, Shattuck Eidens, et al. 1994). The BRCA1 gene product is a negative regulator (tumor suppressor) of mammary cell proliferation (Thompson, Jensen, Obermiller, Page, & Holt. 1995). Further study has found that BRCA1 and BRCA2 contain a sequence motif homologous to members of the granin family of secretory proteins (Jensen, Thompson, Jetton, et al. 1996). The granin family of proteins include chromogranins and secretagranins which have a widespread neuroendocrine

distribution (Simon & Aunis. 1989). Studies on the structural and biochemical properties of granins show that they all contain multiple adjacent basic residues that are potential proprotein convertase cleavage sites. Some members of granins are confirmed to be members of Ca^{2+} binding proteins believed to be important for protein secretion (Pimplikar & Huttner. 1992; Huttner, Gerdes, & Rosa. 1991). Possible biological functions of granins include: (i) precursors to biologically active peptides such as CgA, a peptide that inhibits glucose-induced insulin release from the isolated pancreas (Iacangelo, Fischer Colbrie, Koller, Brownstein, & Eiden. 1988); (ii) packaging of peptides into secretary granules (Rosa, Hille, Lee, Zanini, De Camilli, & Huttner. 1985); (iii) protein sorting and secretory granule biogenesis (Gerdes, Rosa, Phillips, et al. 1989). Taken together, it is believed that the granin proteins are natural targets of most proprotein convertases such as PC1, which is localized in the secretory granules and is responsible for the cleavage of proteins secreted by the regulated secretory pathway. Recent studies have shown that some of the BRCA1 protein molecules are found in secretory granules (Jensen, Thompson, Jetton, et al. 1996), and this is consistent with the concept that BRCA1, as a homologue to granin proteins, may be targeted by proprotein convertases in secretory granules. In addition, BRCA1 contains 35 and BRCA2 contains 56 potentially active dibasic convertase cleavage sites (Steeg. 1996), further supporting the notion that these breast cancer susceptibly gene products are strong candidates for proprotein convertase targets in breast cancer.

Recent studies have shown that stromelysin-3 (ST_3), a member of the MMPs, is overexpressed in fibroblastic cells of invasive breast carcinomas (Basset, Bellocq, Wolf, et al. 1990). ST_3 is also involved in mammary gland involution (Lefebvre, Wolf, Limacher, et al. 1992). ST_3 plays a role in stromal-epithelial interactions during breast carcinoma progression.

ST_3 protein has four domains, these include an N-terminal signal peptide followed by a propeptide, a catalytic domain containing the zinc-binding site, and a C-terminal haemopexin-like domain (Basset, Bellocq, Wolf, et al. 1990). Further studies have found that a stretch of 10 amino acids located at the junction between the ST_3 pro-and catalytic domains contains an active furin cleavage site. Furin is the first proprotein convertase that is responsible for processing proST_3 to ST_3, the active form. In contrast to other MMPs, ST is the only member to be targeted by proprotein convertases (Santavicca, Noel, Angliker, et al. 1996). Thus, overexpression of proprotein convertases in breast tumor cells may enhance the extracellular processing of proST_3 to ST_3 and further enhance the metastatic process.

4. Research Objectives

It is well known that one of the hallmarks of malignancy is an abnormal autocrine or paracrine growth factor regulatory mechanism (Sporn & Roberts. 1985). In human breast cancer, it is believed that nuclear oncogenes and ovarian hormones are emerging as important mediators and modulators of autocrine and paracrine growth factors (Dickson & Lippman. 1992; Dubik & Shiu. 1988; Dubik, Dembinski, & Shiu. 1987). Examination of defective or overexpressed growth-regulatory genes (oncogenes), growth factors, and ovarian hormones is a very active area of breast cancer investigation. Proprotein convertases , based on their unique property of generating biologically active growth and receptor molecules from their inactive procursors , may have an important role in the progression of breast cancer. To date, breast cancer research has not sufficiently investigated an interplay between proprotein

convertases and growth factors. In our laboratory, as an initial approach towards elucidating the role that proprotein convertases may play in growth regulation of breast cancer, 30 primary human breast cancer specimens, 10 specimens of histologically normal human breast tissues, and 6 human breast cancer lines were used to compare the expression of six known proprotein convertases members. Proprotein convertase, PC4, which is testis specific, was not included in the study. The results have shown that PC1, furin, PACE4 and PC7 mRNAs were detected in both the tumor tissues and breast cancer cell lines. PC5 was only detected in a small percentage (2 / 30) of tumor tissues, while PC2 was not detected in either tumor tissues or cancer cell lines. Messenger RNAs for all the proprotein convertase members were not detected in histologically normal human breast tissues (Cheng, Watson, Paterson, Seidah, Chretien, & Shiu. 1997). Thus, an important question to be asked is: what are the biological targets of proprotein convertases in human breast cancer?

To address this question, my work described in this thesis had two aims. Aim one was to develop a cell model for proprotein convertases overexpression in a human breast cancer cell line. Aim two was to examine the cell model and a control cell line for differences in protein patterns. My project focused on the biological effects of PC1 on MCF-7 human breast cancer cell lines. The following investigations have been performed.

(1) The generation of a MCF-7 cell line capable of elevated expression of mouse PC1. A CMV-mPC1 / Neo plasmid was stably transfected into the MCF-7 cell line, G418 resistant clones selected and tested by northern blot and Southern blot analyses for integrity of the plasmid, and PC1 expression. Immunoprecipitation with rabbit polyclonal anti-PC1 antibody, followed by SDS-polyacrylamide and two dimensional SDS-polyacrylamide gel electrophoresis were carried out, to confirm an elevated level of mPC1

protein was produced in the transfected cell line.

(2) A comparison of protein patterns between the mPC1 overexpressing cell line, and a control G-418 resistant MCF-7 cell line on two-dimensional SDS-polyacrylamide gel was made. Four differences in proteins were noted. Some of the altered proteins may be natural PC1 target proteins in human breast cancer.

MATERIALS AND METHODS

1.PREPARATION OF COMPETENT TG1λ BACTERIA AND TRANSFORMATION

TG1λ was inoculated in 5ml LB broth (1 litre LB broth : 10g bactotryptone, 5 g yeast extract, 10 g NaCl , pH 7.0) and was grown overnight in a shaking incubator (250 rpm) at 37°C. The following morning, 5 ml culture was added to 500 ml LB borth in a large sterile flask and once again incubated till OD_{550} is 0.6. Bacterial pellet was spun down with a JA-10 roter, 5000 rpm, 10 minutes, 4°C . Pellet was resuspended in 200 ml of solution A (20 ml 1 M $CaCl_2$,1 ml 2 M Tris-HCl pH 7.5 , 179 ml ddH_2O) and incubated 4 hours on ice . After centrifugation with the same conditions as above, the pellet was resuspended in 20 ml Solution B (10 ml 1 M $CaCl_2$,0.5 ml 2 M Tris-HCl pH 7.5, 14 ml Glycerol ,75.5 ml ddH_2O) and kept at 4°C overnight. The next morning samples were aliquoted into Eppendorf tubes and at -70 °C in 200 μl aliquots.

Transformation was performed by adding 10~50 ng of CMV-mPC1 / neo plasmid to 200 μl competent TG1λ bacteria and set on ice for 40 minutes. CMV-mPC1 / neo was kindly provided by Dr. Nabil Sedah (Clinical Research Institute of Montreal). Bacteria were then heat shocked at 42°C for 1 minute, then 200 μl of warm LB was added and cells were grown at 37°C for 30 minutes . A range of 50-200 μl were spread on LB agar plates (1.2 g in 100 ml LB broth) containing 25 mg/ml ampicillin stock solution and allowed to absorb for 30 minutes, then inverted at 37°C incubated overnight. The following morning transformed bacterial colonies were picked with a sterile loop, inoculated into 5 ml LB containing 9 ul ampicillin stock (25 mg/ ml) and incubated at 37°C, at 250 rpm for 4-6 hours. Stocks were obtained by aliquoting 500 ul of bacteria with 500 λ sterile glycerol and stored at -70 °C.

2. PLASMID DNA AMPLIFICATION AND PURIFICATION

Following bacterial amplification , plasmids were isolated from transformed bacteria and purified by CsCl density gradient centrifugation as described by Maniatis et al (Maniatis, Fritch. 1982). A 5-ml LB containing 9 μl Ampicillin stock (25 mg / ml) was incubated with transformed bacterial stock for overnight. The following morning, plasmid were isolated using the Magic Minipreps (Promega). The plasmid DNA was digested with the appropriate restriction enzyme and electrophoresed on a 1 % agarose gel. Upon identifying the correct plasmid, the remaining bacterial culture was added to a 2000 ml flask containing 500 LB with 900 μl Ampicillin stock (25 mg / ml) and incubated in a shaker incubator. After 4 hours the culture was measured till OD_{600} is 0.6. At this stage the plasmid were amplified by adding 200 μl chloramphenicol stock (34 mg / ml in 100 % ethanol). Allow the cells to grow overnight.

The bacterial culture was centrifuged in a 500 ml Beckman tubes in a JA - 10 roter for 10 minutes at 8000 rpm and 4°C. The pellets were resuspended in 9 ml of cold lysis buffer (25 mM Tris-HCl pH 8.0, 10 mM EDTA, 50 mM glucose). As soon as transferred to 40 ml Oakridge tubes, 1ml of freshly prepared chicken egg white lysozyme (20 mg / ml in lysis buffer) was added and cells were shaken on ice for 30 minutes. Then 10 ml 0.2 M NaOH / 0.2 % SDS solution was added and cells were shaken on ice for 30 minutes. Ten ml 3 M KAC solution (pH 5) was added and cells were again shaken on ice for 30 minutes. Bacterial debris was removed by centrifugation at 18,000 rpm (4°C) for 30 minutes and supernatant was then transferred to 50 ml centrifuge tubes. The supernatant was extracted with phenol for 10-15 minutes. The DNA pellet was resuspended by vortexing in a Ti75 centrifuge tube. Ethidium Bromide (EtBr) (0.2 ml of a 1 mg / ml solution) was overlaid and the remaining

volume was filled with mineral oil. The tubes were balanced, heat sealed, and centrifuged in a Beckman Ti75 rotor at 55,000 rpm for 16 hours, then at 45,000 rpm for 45 min. (both speeds were at 25° C). The plasmid band was identified with UV light and removed carefully with an 18 - gauge needle and 3 ml syringe without disturbing the upper bacterial genomic DNA. The EtBr was removed by 2 - 3 extractions with 5 ml isoamyl alcohol. Finally the plasmid DNA was precipitated with 2.2 volumes of ethanol and 1 / 15 vol 3 M NaAc (pH 5.2, -20° C), centrifuged for 20 min. at 10,000 rpm in a JA-20 rotor, vacuum dried and then redissolved in ddH_2O. DNA was quantitated spectrophometrically by measuring the absorbency at 260 nm.

3.TRANSFECTION AND CELL CULTURE

Transient transfection

The MCF-7 human breast cancer cell line was maintained at 37° C in a humidified atmosphere of 5 % CO_2 in air. Cells were seeded at a density of 1 x 10^6 cells per 10 cm tissue culture dish, in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10 % (V/V) fetal bovine serum, glucose (3.5 g / litre) and Penicillin (100 u / ml). Full length mPC1 in the expression vector pRC-CMV (Invitrogen) containing a gene for neomycin resistance was kindly provided by Dr.Nabil Seidah (Clinical Research Institute of Montreal), and was purified as described in the previous section. Transfection was performed 24 hours after MCF-7 was inoculated. Cells were washed 10 ml of 5 % FBS medium instead of normal 10 % FBS before 2.5 M $CaCl_2$ coprecipitation with CMV-mPC1 (20 μg / dish) and 2 x BBS (pH 6.95). Plasmid DNA pSV-Neo (20 μg / dish) was used as a control. RNA was isolated 36 hours after transfection.

Stable transfection

The transfection steps were essentially the same as those used in the transient transfection, with only one difference-both CMV-mPC1 / Neo and pSV-NEO were linerized with restriction enzyme ScaI. Twenty-four hours after transfection , cells were rinsed with PBS twice, then 10 % FBS fresh media were added for another 24 hours. The following day cells were split at (1:10) and incubated an additional 24 hours before applying selection for stable transfection . Cells with stable plasmid integration were selected in 10 % FBS containing 1 mg / ml of a neomycin analog G418. After approximately three weeks single clones were isolated with cloning rings, and passaged into 96 well plates, then into 24 well plates, then 6 well plates , then into 10 cm plate. Clones surviving the initial selection and passage into 6 well plate usually survived the entire coloning process. All clones positive for mPC1 mRNA were preserved with 10 % DMSO in 10 % FBS in liquid N_2 .

4. RNA ISOLATION AND NORTHERN BLOT ANALYSIS

Cellular RNA was extracted by homogenizing in a guamidinium thiocyanate solution (GuSCN 4 M guanidine thiocyanate, 17 mM N-lauroyl sarcosine, 0.007 % 2-mercaptoethanol, and 25 mM sodium citrate pH 7.0), aspirated through a 18 gauge needle in order to totally break up the cells, laid over a 5 ml cesium chloride cushion (5.7 M cesium chloride, 0.1 M EDTA pH 7.5) in Beckman Quickseal centrifuge tubes, and then centrifuged at 35,000 rpm for 20 hours in a Ti75 roter as described by Chirgwin et al (Chirgwin, Przybyla. 1979). After centrifugation, the RNA pellet was resuspended in ddH_2O, precipitated with sodium acetate-ethanol (0.2 M NaAc, pH 5.2, 2.2 vol ethanol) at -70° C and once again

pelleted, dried and redessiolved in ddH_2O. RNA was quantitated spectrophotometrically at a wave length of 260 nm. Thirty μg of total RNA per sample (in a ~ 9 μl sample volume) was denatured by adding 20 μl formamide, 7 μl formaldehyde, 4 μl 5 x gel running buffer (GRB : 0.2 M MOPS, 50 mM sodium acetate pH 5.2, 5 mM EDTA pH 7.5), total volume was 40 λ and incubated at 65° C for 15 minutes. The denatured RNA was electrophoresed on a 1 % agarose-2.2 M formaldehyde denaturing gel containing ethidium bromide (0.5 μg / ml) in 1 x GRB as outlined in Maniatis et al (Maniatis, Fritch. 1982), and set at 20~25 volts overnight. The following morning the gel was transferred to a NitroPlus 2000 (Micron Seperation Inc.) membrane in 20 x SSC (3 M NaCl, 0.3 M sodium citrate pH 7) solution overnight. The next morning the nitrocellulose membrane was put in a 80°C oven for two hours in order to immobilize the RNA. After prehybridization for 2 hours in 50 % Formamide/SSPE (20 x SSPE: 3 M NaCl, 0.2 M NaH_2PO_4, 0.025 M EDTA, pH 7.4) prehybrization solution (50 % formamide, 25 % 20x SSPE, 10 % 50x Denhardt's, 3.5 mM SDS, Sheared Salmon Sperm DNA 10 mg / ml) the membrane was hybridized overnight with ^{32}P- labeled CMV-MPC1 probe prepared by Nick Translation (P.R.Langer, and D.C.Ward. 1981). Blots were washed from 2 x SSC (1 x SSC: 0.015 M Sodium Citrate, 0.15 M NaCl, pH 7.0) at room temperature for 15 minutes, twice, to a final stringency of 0.1 x SSC at 65°C for 10-20 minutes., and exposed to Kodak X AR X-ray film at -70°C.

5. DNA ISOLATION AND SOUTHERN BLOT ANALYSIS

MCF-7 cells were washed with phosphate buffered saline (PBS: 8.0 g NaCl, 0.2 g KCl , 1.5 g NaH_2PO_4, 0.2 g K_2HPO_4 per litre of solution), scraped from a 15 cm cell culture

plates using a rubber policeman, then washed again with PBS before being pelleted and stored at -70°C. Genomic DNA was isolated using Easy-DNA™ Kit (Invitrogen) from frozen cell pellets. Finally DNA was redissolved in ddH_2O.

Southern analysis was performed on 0.8 % agarose gels following digestion of genomic DNA with PSTI restriction endonuclease. Usually 10 μg of DNA was digested overnight with 30 units of PSTI, then size-separated by electrophoresis on a 0.8 % agarose gel (in 1 x TBE) overnight at 20-30 volts. Southern transfer of genomic DNA was as described by Maniatis et al (Maniatis, Fritch. 1982). Before transfer the gel was denatured in a 0.5 M NaOH, 1.5 M NaCl solution for 30 minutes twice with gentle agitation and neutralized in 1 M NH_4Ac-20 mM NaOH for another 30 minutes twice. Hybridization was carried out with the same process as Northern, but the ^{32}P-probe was the purified mPC1 insert instead of the whole plasmid.

6. CELL LABELING, IMMUNOPRECIPITATION, AND ONE- AND TWO-DIMENSIONAL SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

A chosen cell line was taken from N_2 liquid and cultured in a 10 cm culture dish for 2~3 days before labeling. After washing three times in cysteine-free Dulbecco's modified Eagle's medium with 2 mM L-Glutamine, Penicillin 100 u / ml and 3.5 g / liter glucose, the cells were labeled with 100 μCi / ml L-Cysteine ^{35}S (ICN, 800 Ci / mmol) for 24 hours at 37°C, 5 % CO_2 incubator. The medium was collected and centrifuged at 800 rpm, 5 minutes, room temperature to remove cellular debris. Cells were washed three times with cold phosphate-buffered saline and removed from the dish with a rubber policeman. After centrifuged at 800 rpm, 5 minutes, room temperature cell pellets were stored at -20°C.

Conditioned medium was dialyzed for 24 hours in a Spectrapor membrane tubing (number 3, 15 mm x 50 ft,mw cut off: 3500, Spectrum Medical Industries, INC) and lyophilized.

Lyophilized conditioned medium was dissolved in 50 to100 μl IPB containing 1 % Triton X-100 and 1 % Trasylol (Aprotinin) (10,000 K.I.U./ ml, Miles Pharmaceuticals). Cell lysate was aspirated through a 21 gauge needle in 50~100 μl of the above solution and centrifuged at 35,000 rpm, 4°C for 2 minutes to get rid of cellular debris. In order to immunoprecipitate mPC1 protein, 1 μl rabbit anti-PC1 (C- terminal) (provided by Dr. NabilG. Seidah, laboratory of Biochemical Neuroendocrinology of Clinical Research Institute of Montreal) was added to 50 μl of either cell lysate and medium. Normal rabbit serum was used as control. After incubation at 4°C overnight, 10 μl of washed Pansorbin slurry (fixed S. aureas containing Protein A, from Calbiochem) was add. Immunoprecipitates were washed successively with 1 ml IPB , 1 ml IPB plus 3M urea, and 1 ml IPB. Washed immunoprecipitates were solubized in SDS- PAGE sample buffer (25 mM phosphate buffer, pH 7.0, 2 % SDS, 10 % (v / v) glycerol, 5 % 2-mercaptoethanol, trace of bromophenol blue) at 100°C for 5 minutes, pellets were removed by centrifuged at 35,000 rpm, 4°C, 5 minutes. Samples were subjected to SDS-PAGE (3 % stacking gel ; 10 % resolving gel), and gels were soaked in staining solution 30 min. (7 % acetic acid, 20 % methonal, 0.05 % commasie blue), by destaining for 30 minutes (7 % acetic acid, 10 % methanol), fluorography was done by soaking the gel in DMSO 1.5 hours (dimethyl sulfoxide), 20 % PPO in DMSO 3.5 hours, and 1 liter ddH_2O. The impregnated gel was dried under vacuum and autoradiographed on preflashed Kodak X-Omat AR X-ray film.

To perform two- dimensional gel electrophoresis both cell lysate and lyophilized medium were dissolved in 50-100 μl lysis buffer (9.5 M urea, 2 % (w / v) NP-40, 2 %

Ampholines pH 3.5-10, 5 % β-mercaptoethanol). Isoelectric focusing in the first dimension was performed in the presence of urea 8.25 g, ddH_2O 6 ml, 30 % Acrylamide / 1.8 % Bis 2 ml, Ampholine (pH 3.5-10) 750 μl, NP-40 300 μl, TEMED 10 μl, 70 μl of 10 % APS as described by O' Farrell (O'Farrell. 1975), and samples were loaded with equal radioactivity according to TCA precipitation. Fresh 2.5 hours degassed 0.02 M NaOH were as upper buffer, and the total voltage and time (in hours) were around 10,000 volt hours (volts x hours). Gels were soaked in SDS sample buffer (10 % w / v glycerol, 5 % v / v β-mercaptoethanol, 2.3 % w / v SDS, and 0.0625 M Tris-HCl, (pH 6.8) and stored in - 20°C.

SDS-Polyacrylamide gel electrophoresis in the second dimension was carried out on a slab with 3 % stacking gel and 15 % resolving gel. The conditions were identical to those used from one-dimensional SDS-gel electrophoresis.The Immunoprecipited proteins were also analyzed by two- dimensional polyacrylamide gel electrophoresis after redissolved in 20 mM Tris-HCl (pH 7.4), 8 M urea, 5 % TritonX-100, 1 % 2-mercaptoethanol, 2 mM EDTA instead of in SDS-PAGE or lysis buffer.

RESULTS

1. THE EXPRESSION OF MOUSE CONVERTASE MPC1 IN HUMAN BREAST CANCER MCF-7 CELLS

A full-length mouse PC1 cDNA had previously been inserted into the eukaryotic expression vector pRC / CMV which contains an enhancer / promoter sequence from the immediate early gene of human cytomegalovirus (CMV) for high level transcription, the polyadenylation signal and transcription termination sequences from the bovine growth hormone gene, the ampicillin resistance gene and the neomycin resistance gene (Neo), Fig (4). The CMV-mPC1 construct was provided by Dr. Seidah (Clinical Research Institute of Montreal) and has been shown to produce a functional PC1 protein upon transient transfection into several cell lines, including AtT20 pituitary tumor cells (Seidah, Chretien, & Day. 1994). For my experiments the CMV-mPC1 was first introduced into wild type MCF-7 cells through transient transfection. High expression of mPC1 mRNA was confirmed by northern blot hybridization (Fig.5). These results established that the PC1 construct functioned as expected in MCF-7 cells.

As my main aim was to study the biological action of PC1 in a human breast cancer cell line, it was necessary to establish stable transfectants to approch this goal. The linearized CMV-mPC1 plasmid was stably transfected into wild type MCF-7 cells. Fourteen G418 resistant colonies were transferred into a 96-well dish. Of the fourteen clones isolated, eight survived the selection process. Stable clones were screened for expression of mPC1 mRNA by northern blot hybridization and two of the eight clones were found to express mPC1

mRNA (Fig.5). One prominent 2.5 kb RNA species was seen in clone 6, when 30 μg of total RNA from each clone was hybridized with a ^{32}P-CMV-mPC1 probe on a northern blot. This 2.5 kb band is in agreement with the transcript size from the CMV-mPC1 expression vector detected in the transient transfection results and has been previously determined for mPC1 (Fig. 5)(Seidah, Gaspar, Mion, Marcinkiewicz, Mbikay, & Chretien. 1990). The endogenous human PC1 transcript in the wild type MCF-7 and the remaining 6 G418 resistant clones was not detected.

Genomic Southern hybridization experiments were performed in order to determine whether the expression difference between clone 2 and 6 were due to the intact or the rearrangement of mPC1 DNA in the chromosome. Genomic DNA was isolated from all established clones and digested with the restriction endonuclease PstI which can give a distinct pattern between mPC1 insert and CMV vector (Fig. 6). An expected 2 kb and 400 bp band, were present in clone 6 (Fig. 7). However, a major unexpected 1.5 kb band as well as the expected 2.0 kb band which was present in only a minor amount, were detected in clone 2, the low expression clone. It appears that the 1.5 kb may represent a truncated form of the insert construct which was unable to express the mPC1 transcripts, while the minor 2.0 kb band may help to explain the low level of mPC1 transcripts. The Southern blot suggests there are multiple copies of the truncated form present.

The morphology of clone 6 compared to wild type MCF-7 was different. Wild type MCF-7 cells seemed more like epithelium cells, and clone 6 seemed different with lots of branches out of the cell bodies(Fig 8).

2. IMMUNOPRECIPITATION OF CELLULAR EXTRACTS AND CONDITIONED MEDIA

In order to verify that the mPC1 mRNA was translated and was able to produce the active protein product, immunoprecipitation was carried out on clone 6, which expresses higher mPC1 mRNA levels compared to clone 2. Clone 6 was labeled with ^{35}S-Cysteine and cellular extracts and conditioned media were collected after 24 hours as described in Methods. Immunoprecipitation was performed using rabbit anti-mPC1 antiserum, and normal rabbit serum as an control. Staphylococcal Protein A (Pansorbin) described by Kessler (Kessler. 1975) was used as the antibody adsorbent. Immunoprecipitates were dissolved in SDS-Sample buffer and were electrophoresed on a discontinuous gel with a 3 % stacking and a 15 % resolving gel. An expected mPC1 band of approximately 120 kDa was observed both in clone 6 cell lysate and medium when antiserum was used (Fig.9). This correlated with the reported PC1 protein size (Seidah, Marcinkiewicz, Benjannet, et al. 1991).

In order to determine the degree of glycosylation of the mPC1 expression in clone 6, two-dimensional electrophoresis gel was performed on the immunoprecipitated protein. mPC1 proteins were resolved into four species with pIs from 5.58 to 6.17; these likely arise from different degrees of glycosylation (Fig.10). Normally, the mature mPC1 protein consists of 726 amino acids, with three glycosylation sites (Seidah, Marcinkiewicz, Benjannet, et al. 1991).

3. TWO-DIMENSIONAL GEL COMPARISON OF CLONE PROTEINS EXPRESSED IN mPC1-6 AND CONTROL CELL LYSATES

Once an mPC1 stable transfected MCF-7 cell line was isolated (Aim 1), the second step (aim 2) was to identify potential PC1 target proteins in human breast cancer cells; this was the main aim of my project. Although there were several ways to approach the above goal, I selected two-dimensional gel electrophoresis for this analysis. Two-dimensional gel electrophoresis is the combination of two high-resolution electrophoretic procedures, (isoelectric focusing and SDS-polyacrylamide gel electrophoresis) to provide an extremely powerful analytical tool for the characterization of complex protein mixtures from whole cell lysates (O'Farrell. 1975). Since charge and molecular weight are monitored simultaneously, small alterations in proteins can be detected. No other procedure can resolve so many proteins in a single operation. Therefore, two-dimensional electrophoresis is one of the best ways to examine protein changes between control and experimental samples. With very few exceptions, each spot on a two-dimensional gel contains only one protein, whereas a band on a one-dimensional gel may contain more than one protein Single proteins can, however, yield multiple spots on two-dimensional gel due to variability in glycosylation as seen in my results in Figure 10. In addition to analytical applications, two-dimensional gel electrophoresis can provide a means of collecting small amounts of extremely pure protein for amino acid sequence analysis (Hirano. 1989) or antibody production (B.S.Dunbar. 1987).

Two-dimensional gel analysis was used to compare cellular proteins expressed in clone 6 and clone 7; this latter clone was generated as a Neomycin-resistant clone but does not contain an integrated mPC1 construct (Fig.5). In addition, clone 6 was compared to clone-Neo, a neomycin-resistant MCF-7 generated three years ago by another graduate

student (Amir Ashique) in this labaratory.

One experiment comparing clone 6 and clone-Neo (Fig 11-12), and three separate experiments comparing clone 6 and clone 7 (Fig 13-18) were performed in order to identify potential differences between the expressed proteins in the presence or absence of mPC1. Cells were labeled with ^{35}S-Cysteine as described; proteins were initially collected at 12 hours and 24 hours, and both cell lysate and cell media proteins were loaded on two-dimensional gels. The main aim of my project was to identify any consistant and reproducible differences in protein patterns in each separate experiment; I found that there was no difference in protein patterns in two-dimensional gel between protein samples collected at 12 hours and 24 hours; the data reported are for only 24 hours from the following experiments. In addition, there was no reproducible protein patterns differences in two-dimensional gel between cell media as compared to cell lysate; therefore all the results shown are only cell lysate.

Four reproducible differences in protein spots (a, b, c, d) were identified (Figures 11 to 18). The molecular weight and pI of each protein spot were as follows: a, 17 kDa, pI 4.8; b, 12 kDa, pI 5.3; c, 20 kDa, pI 5.8; d, 12 kDa, pI 6.4. In the four independent experiments, consistent differences between mPC1-6 transfectant and control cells were seen as follows: protein c was consistently decreased while proteins a and b were consistently elevated in expression. Protein d was elevated in the mPC1-6 transfectant in two of the experiments. Changes of these four proteins in the four experiments are analysed in Table 2.

DISCUSSION

It is well known that the pivotal role of growth factors in hormone-regulated cancers is the basis of the altered autocrine or paracrine models of cell proliferation (Sporn & Roberts. 1985). In human breast cancer not only have growth factors and their receptors been found in abnormal amounts, but also it has been possible to generate this phenotype by overexpression of such factors by gene transfection. Therefore the processing of biologically active growth factors and their receptors may play a key role in controlling the growth behavior of cancer cells. Proprotein convertases, a class of enzymes involved in posttranslational processing of biological active growth factors and receptor molecules, may therefore be important contributing factors in tumorigenesis and malignant transformation. A complete understanding of the biological function of proprotein convertases in cell growth regulation may provide relevant therapeutic strategies to inhibit malignant cell growth. To this end, I have generated a breast cancer cell model capable of over-expressing one of the convertases, PC1.

In the present study I have stably transfected a CMV-mPC1 / Neo construct into MCF-7 human breast cancer cells. I have confirmed that one clone, clone 6, expressed not only mPC1 mRNA but also the authentic protein. Another G418 resistant cell line, clone 7, has been obtained which did not express PC1 mRNA or protein. Thus, any differences between these two closely related cell lines are likely due to the consequence of PC1 action. In order to compare biological differences between PC1 transfected and untransfected cell lines, proteins from cell lysates, or conditioned media were analyzed by two-dimensional SDS-polyacrylamide gel after clone 6 and clone 7 were labeled with ^{35}S-Cysteine. There

were four reproducible protein spots that were different between these clones. Our present hypothesis of how the four proteins might have been generated is depicted in Fig 19. This was based on the molecular weights of each of the protein spots and on whether the amounts of the spots increased or decreased. These proteins appear to be targets of natural proprotein convertase PC1, and they may reflect a direct linkage between proprotein convertases and human breast cancer. To date, there are no reports to elucidate the relationship between proprotein convertases and human breast cancer. Only when these proteins are microsequenced can we begin to understand the real biological target of PC1 in a human breast cancer cell line. However, there are several possible PC1 target candidates we can think about.

Two possible PC1 target proteins are EGF and TGF-β, two growth hormones that are important in human breast cancer progression (Dickson & Lippman. 1995; Dubois, Laprise, Blanchette, Gentry, & Leduc. 1995). Sequence analysis of EGF has shown several proprotein convertase cleavage sites; the molecular weight of EGF protein is 6 kDa, compared to the the molecular weights of the four protein spots (12 kDa, 17 kDa, 20 kDa) which were identified in this study. EGF therefore does not appear to be a PC1 target candidate in these cells.

Experiments have confirmed that human furin plays a major role in converting the 55kDa pro-TGF-β_1 to its biologically activate form (Dubois, Laprise, Blanchette, Gentry, & Leduc. 1995). The molecular weight of biologically activate form of TGF-β_1 is 12.5 kDa, similar to that of the protein spots b and d (12 kDa) (Dubois, Laprise, Blanchette, Gentry, & Leduc. 1995). TGF-β_1 is therefore a possible PC1 target in MCF-7 cell lines. TGF-β_1

antibody can be used to immunoprecipitate and detect on a two-dimensional gel to test this possibility further.

BRCA1, a human breast cancer suppressor gene, which has 35 potential proprotein convertase cleavage sites, is a possible PC1 target. In a preliminary experiment in our laboratory, we have been able to use C-20 (C-terminal antibody of BRCA1) successfully to immunoprecipitate BRCA1 as a 220 kDa protein from ^{35}S-Cysteine labeled MDA-MB-468 cell lysate and medium (data not shown). MDA-MB-468 is a hormone independent human breast cancer cell line; this cell line has been reported to express the highest level of BRCA1 among the many human breast cancer cell lines tested (Jensen, Thompson, Jetton, et al. 1996). In addition to the intact 220 kDa BRCA1 band, one larger band and several small peptides were also specificially precipitated by C-20, suggesting that some of the smaller peptides may be cleavage products of BRCA1 (data not shown). In my experiment I have tried several times to use C-20 to immunoprecipitate BRCA1 in PC1 overexpressing MCF-7 cell lines; only a minor 220kDa band was observed. This correlates with a previous report that endogenous PC1 in the MCF-7 cell line was less as compared to MDA-MB-468 cell line (Holt, Thompson, Szabo, et al. 1996). The results with clone 6 do not suggest that BRCA1 is among the proteins identified in this study. A BRCA1 antibody can be used to detect on two-dimensional gel to further answer the above question.

A recent report shows that stromelysin-3, an enzyme which belongs to the matrix metalloproteinase family, is expressed in most invasive human carcinomas, and mammary gland involution. One of the proprotein convertases, furin, has been confirmed to play a major role in converting pro-stromelysin-3 to its biologically activate form in the MCF-7 cell line

(Santavicca, Noel, Angliker, et al. 1996). The molecular weight of biologically activate form of stromelysin-3 protein is 47 kDa, which is larger than the molecular weight of the four protein spots. Thus, stromelysin-3 is likely not a PC1 target in these cells.

In the two-dimensional gel experiments, there are still some potential pitfalls. First, not all proteins are soluble in urea, and those proteins not soluble in urea would not be detected. Second, many higher molecular weight proteins were not well resolved, and these proteins would have escaped comparison. Third, the cells were labeled with ^{35}S-Cysteine for 24 hours, and this procedure may have missed proteins with low Cysteine composition. Methionine is another amino acid I could have used for labeling proteins.

By comparing protein spots in two-dimensional SDS-polyacrymide gel between clone 6 and clone 7, and between clone 6 and clone-Neo, four consistent and reproducible changes in protein spots were observed. Thus, overexpression of PC1 in MCF-7 cell line appears to have altered the host cell protein patterns. One important aspect of the future work will be to perform microsequencing of these protein spots to establish the identity of these proteins and if these proteins are related as hypothesized in Figure 19. After microsequencing, the known amino acid sequences could be used to make synthetic oligonucleotide probes for the eventual cloning of these mRNAs.

It is possible that the new protein spots have arisen from new gene expression, either due to integration of the CMV-mPC1 or to activation of host genes by the transfected mPC1. An overexpression of a proprotein convertase may change the microenviroment of the nucleus by either activating or inhibiting some factors such as transcription factors, and this may indirectly affect the synthesis of the four protein spots. One way to address this question is

to co-transfect clone 6 with an inhibitor of proprotein convertases, such as α_1-PDX. If the proteins (a, b, c, and d) were PC1 targets, then they should not be produced in clone 6 transfected with α_1-PDX (Angliker, Wikstrom, Shaw, Brenner, & Fuller. 1993). In addition, I could look at other transfected clones to further confirm the protein spots.

In the future clone 6 and clone 7 will be useful in the following proposed studies: the effect of convertase on cell growth. It is possible that overexpression of PC1 alone will not alter cellular functions enough to produce discernable changes in cell culture study, but clone 6 and clone 7 may be useful when we study the growth *in vitro* of transfected MCF-7 cells heterotransplanted into immunodeficient athymic nude mice. The growth in athymic nude mice of MCF-7 cells with altered expression of convertases could be compared to control cells according to the published procedures (Leung C.A.,Shiu, R.P.C. 1981). These studies may lead to novel insights into the biological function of proprotein convertases in human breast cancer.

Table 1 Proprotein Precursors Cleaved By Furin

Viral glycoproteins and proteins

Human cytomegalovirus glycoprotein B

Measles virus glycoprotein F_0

Newcastle disease virus glycoprotein F_0

Human immunodeficiency virus glycoprotein 160 (gp 160)

Fowl plague (influenza A) virus hemaggkutinin

Sindbis virus gpE_2

Mouse mammary tumor virus-7 superantign

Human parainfluenza virus type 3 glycoprotein F_0

Toxins

Shigella dysenteria type 1 shiga toxin subunit A

Corynebacterium diphtheriae diphtheria toxin

Bacillus anthracis protective antigen (PA)

Pseudomonas aeruginosa exotoxin A

Receptors

Human insulin pro-receptor

Hepatocyte growth factor / scatter factor receptor (HGF / SF)

Plasma proteins

Human proalbumin

Human complement pro-C3

Human pro-von Willebrand factor

Human pro-factor IX

Human vitamin K-dependent pro-factor X

Hormones and growth factors

Human pro-parathyroid related peptide

Human pro-parathyroid hormone

Mouse pro-β-nerve growth factor

Human pro-transforming growth factor typeβ

Human pro-endothelin-1 (pET-1)

Others

Human pro-furin

Human stromelysin-3

Table 2

Exp. \ protein spots	a	b	c	d
Exp. .1 Clone 6 vs. Clone-Neo (Fig. 11 + 12)	↑	↑	↓	↔
Exp. .2 Clone 6 vs. Clone 7 (Fig. 13 + 14)	↑	↑	↓	↑
Exp. .3 Clone 6 vs. Clone 7 (Fig. 15 + 16)	↑	↑	↓	↑
Exp. .4 Clone 6 vs. Clone 7 (Fig. 17 + 18)	↑	↑	↓	↔

The four consistant and reproducible protein spots in four experiments were analysed according to the changes of protein expressions, and ↑ means increase; ↓ means decrease; and ↔ means no change.

Figure 1. Hierarchy of growth-regulatory molecules. {29}

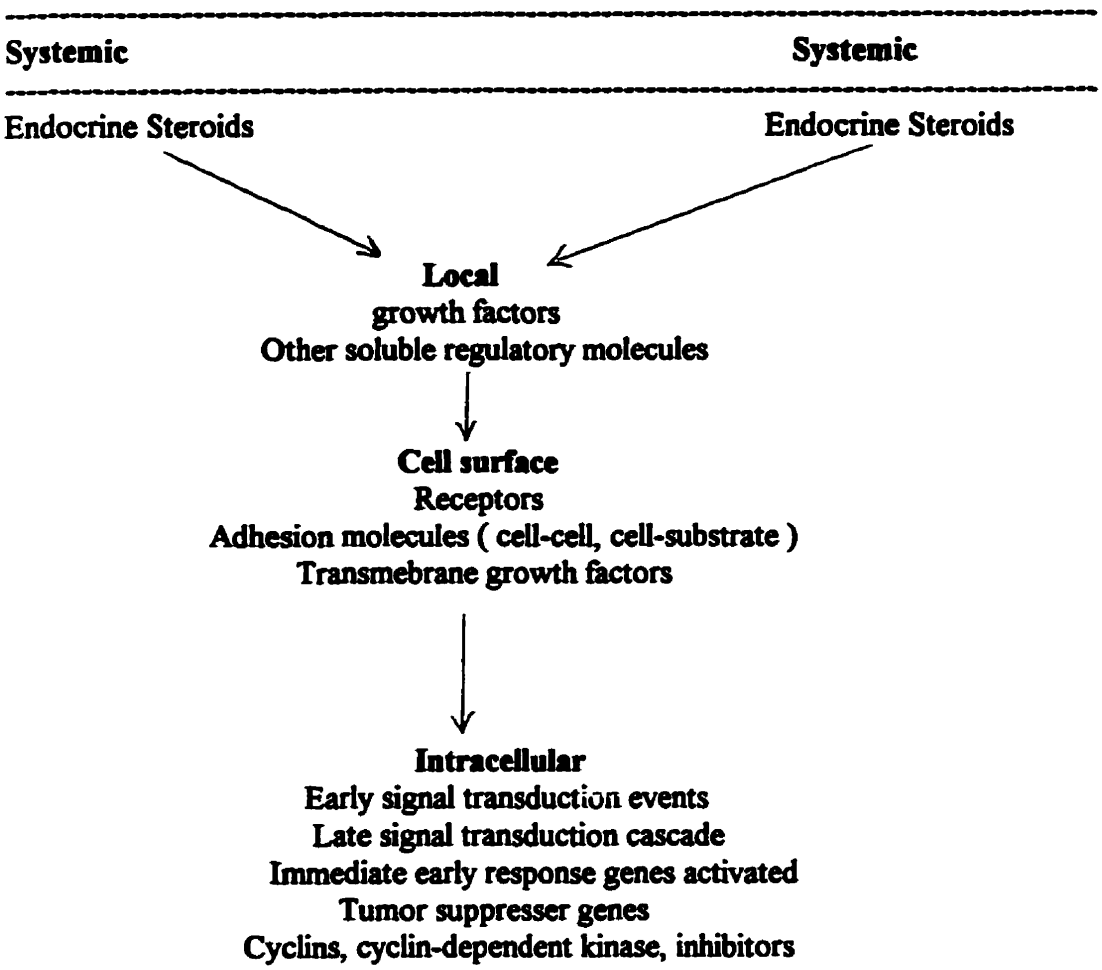

Systemic
Systemic
Endocrine Steroids
Endocrine Steroids
Local
growth factors
Other soluble regulatory molecules
Cell surface
Receptors
Adhesion molecules (cell-cell, cell-substrate)
Transmebrane growth factors
Intracellular
Early signal transduction events
Late signal transduction cascade
Immediate early response genes activated
Tumor suppresser genes
Cyclins, cyclin-dependent kinase, inhibitors

Figure 2. Functional interactions between sex steroids, growth factors, oncogenes, and tumor suppressor genes in epithelial cell cycle in breast cancer. {29}

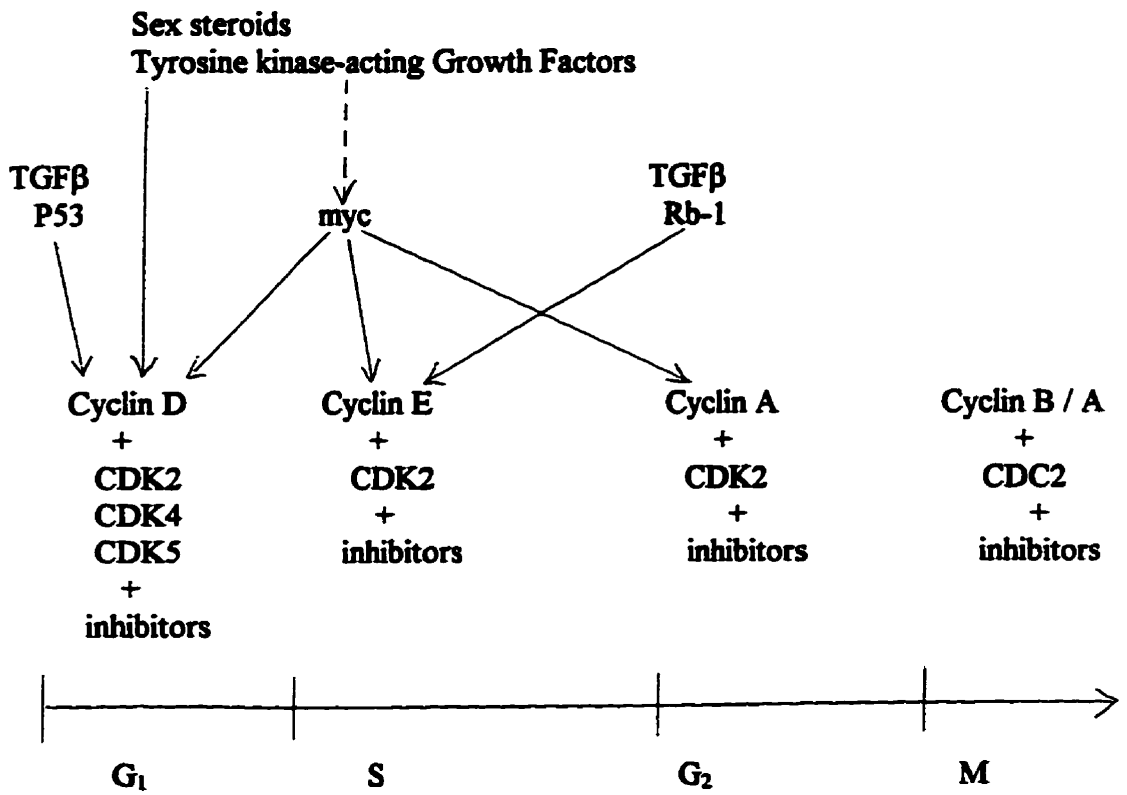

Sex steroids
Tyrosine kinase-acting Growth Factors
TGFβ
P53
myc
TGFβ
Rb-1
Cyclin D
+
CDK2
CDK4
CDK5
+
inhibitors
Cyclin E
+
CDK2
+
inhibitors
Cyclin A
+
CDK2
+
inhibitors
Cyclin B / A
+
CDC2
+
inhibitors
G1
S
G2
M

Figure 3. A summary of the structure of the six mammalian proprotein convertases.

{124}

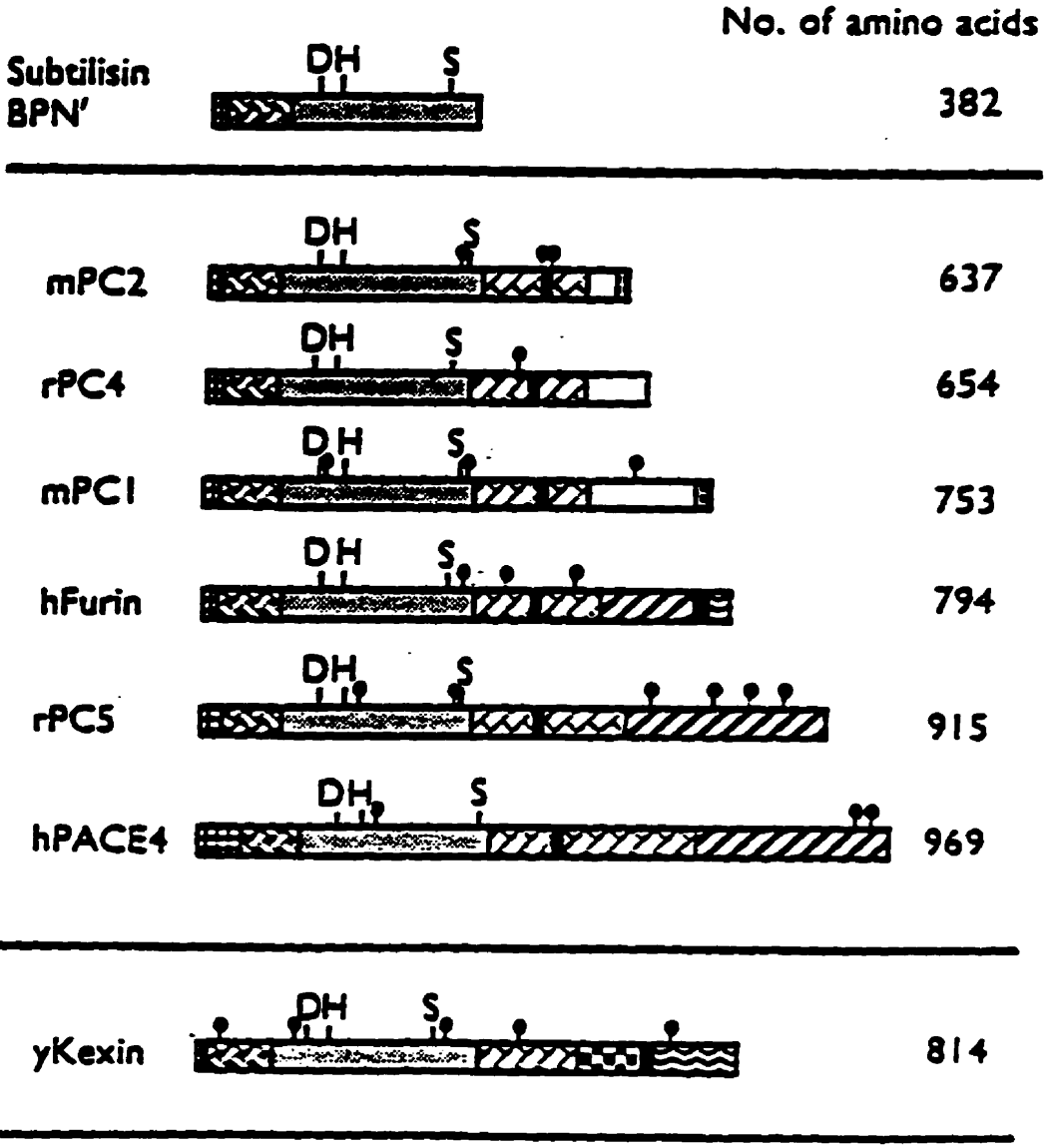

No. of amino acids
Subtilisin BPN'
DH
S
382
mPC2
637
rPC4
654
mPC1
753
hFurin
794
rPC5
915
hPACE4
969
yKexin
814
Signal Peptide
Pro-Segment
Catalytic
P-Domain
Transmembrane
Cytoplasmic
RGD
Ser/Thr
Amphipathic
Cys-rich
N-Glycosylation

Figure 4. The CMV-mPC1 construct

The full-length mouse PC1 cDNA was previously inserted into the eukaryotic expression vector pRC / CMV (provided by Dr. Seidah, Clinical Research Institute of Montreal), which contains an enhancer / promoter sequence from CMV for high level transcription. The vector also contains the polyadenylation signal and transcription termination sequences from the bovine growth hormone gene, the ampicillin resistant gene and Neomycin resistance (Neo) gene.

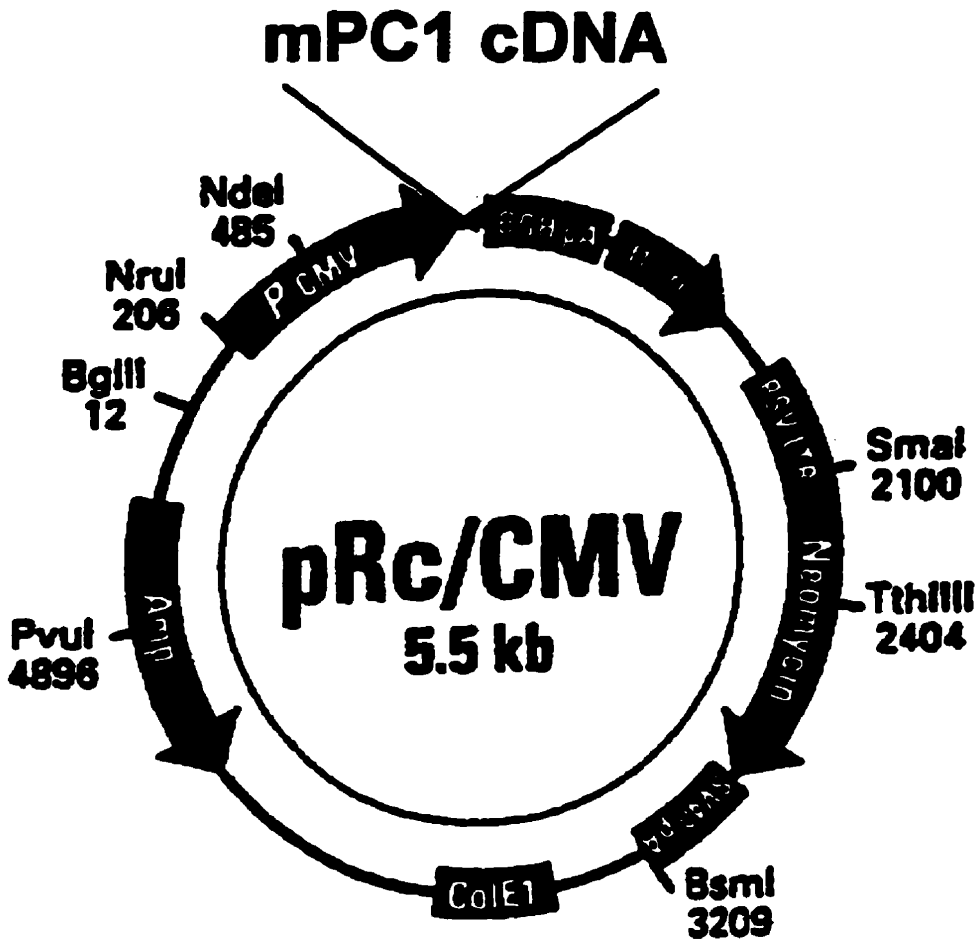

mPC1 cDNA
NdeI
485
NruI
206
BglII
12
PvuI
4896
SmaI
2100
TthIII
2404
BsmI
3209
pRc/CMV
5.5 kb
P CMV
ColE1

Figure 5. Northern blot analysis of mPC1 mRNA expression in MCF-7 clones. TR (transient transfectants), SR (stable transfectants).

Eight G418 resistant clones (lanes marked 1, 2, 3, 6, 7, 9, 13, and 14) survived the selection process when the linearized CMV-mPC1 plasmid was transfected into MCF-7 cell line. 30 μg total RNA from each clone was electrophoresed on an agarose formaldehyde gel, blotted onto a nitrocellulose, and hybridized with an ^{32}P-CMV-mPC1 probe. One band of 2.5 kb which was much more prominent in clone 6 than clone 2.

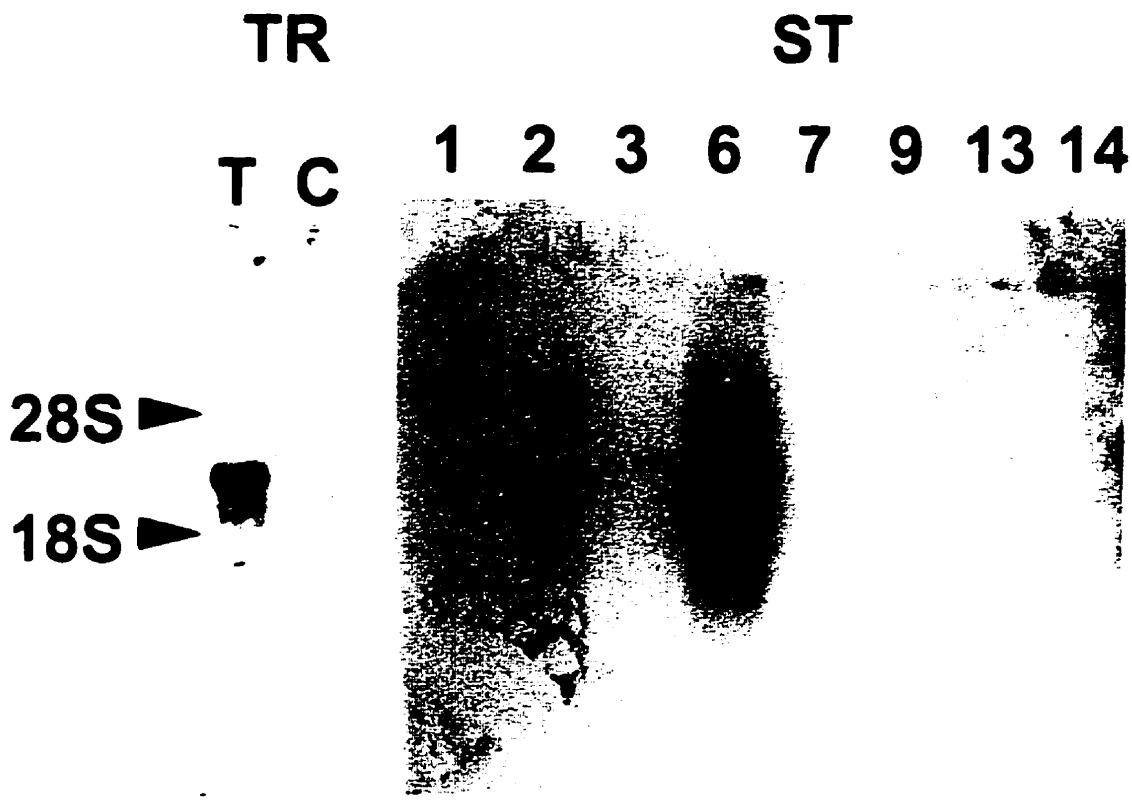

TR
ST
T C
1 2 3 6 7 9 13 14
28S
18S

Figure 6. Map of PstI digestion site in plasmid CMV-mPC1.

PstI restriction enzyme digests twice in both mPC1 cDNA insert and CMV vector. ScaI restriction enzyme was used to linearize CMV-mPC1 before stable transfection. Upon integration into the host genome, and PstI digestion of genomic cDNA, the 400 and 2000 bp bands are expected to hybridize to ^{32}P-mPC1 insert on the Southern blot shown in Figure 7. If no rearrangments have occured.

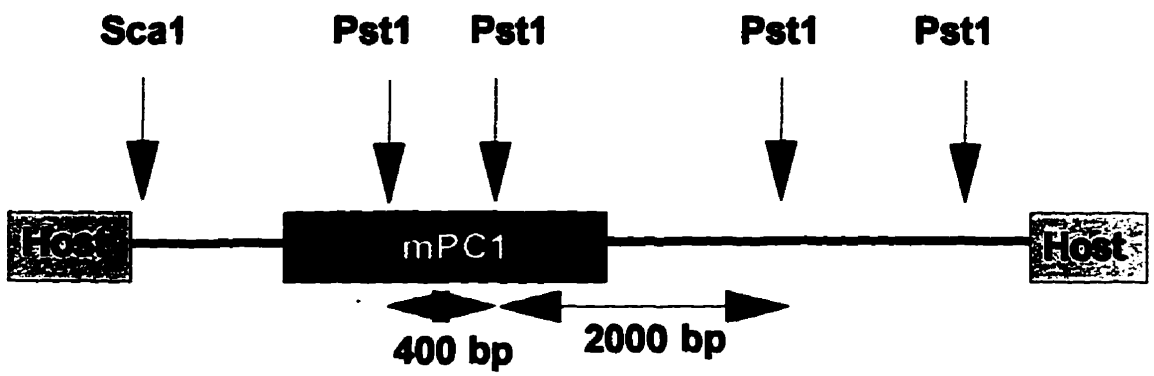

Sca1
Pst1
Pst1
Pst1
Pst1
Host
mPC1
Host
400 bp
2000 bp

Figure 7. Southern blot analysis of mPC1 DNA in clone 2 and clone 6.

Southern analysis was performed with 10 μg of PstI digested genomic DNA from all MCF-7 clones and hybridized with a ^{32}P-labeled mPC1 cDNA probe. As predicted from the restriction enzyme of CMV-mPC1, expected 2 kb and 400 bp band were present in clone 6, a high expressing clone. A major 1.5 kb and a minor 2 kb band were present in clone 2, a low expressing clone. The 1.5 kb band may be due to a truncated form of the insert mPC1. The presence of the minor 2kb band may help to explain the low mRNA expression level of mPC1 in clone 2.

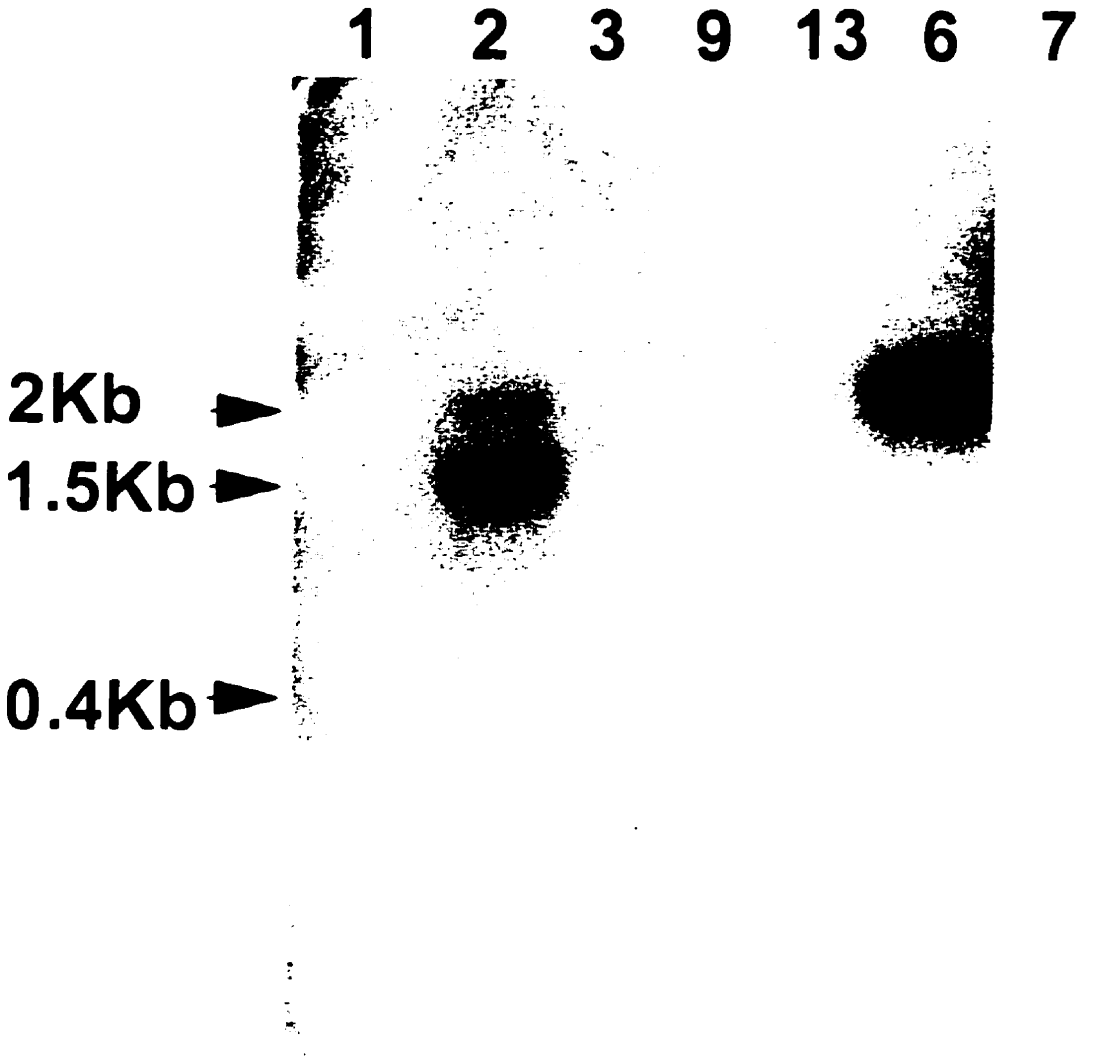

1
2
3
9
13
6
7
2Kb
1.5Kb
0.4Kb

Figure 8. Morphological differences between clone 6 (A) and wild type MCF-7 (B)

Cells were plated with 1 x 10^7 per 10 ml dish and grown for three days before the pictures were taken. Wild type MCF-7 is more like epithelium cells and clone 6 shows more branches out of the cell bodies.

A

B

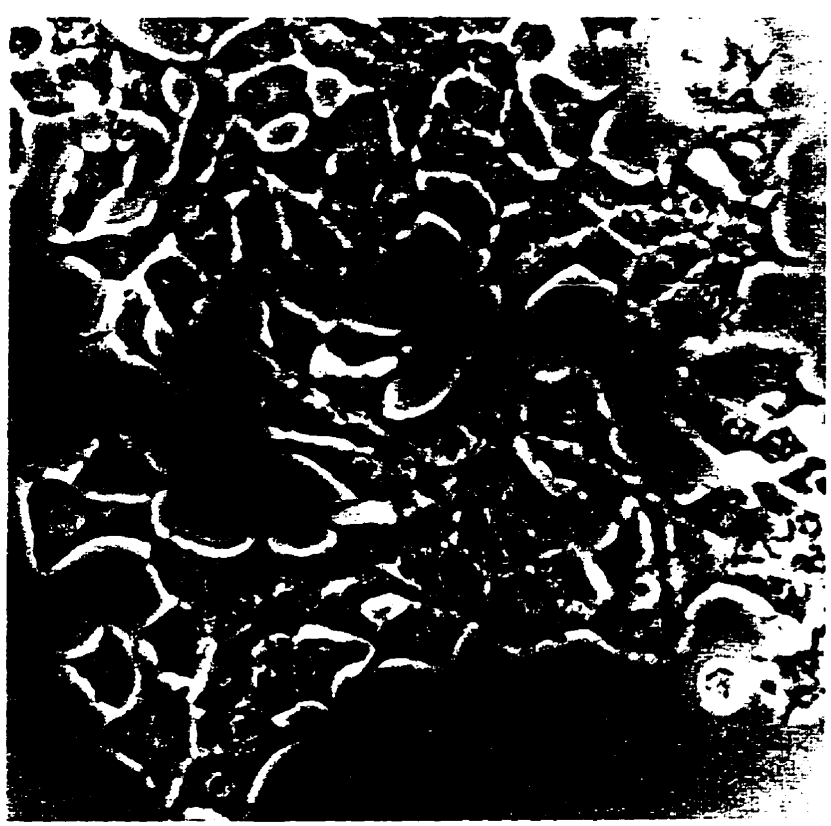


Figure 9. Immunoprecipitation of cell lysate and conditioned medium of clone 6.

Clone 6 was labeled with ^{35}S-Cysteine and both cell lysate and conditioned medium were collected after 24 hours. Immunoprecipitation was performed using rabbit anti-PC1 antiserum, and normal rabbit serum as control. An expected mPC1 band of approximately 120 kDa was observed both in cell lysate and medium, precipitated with anti PC-1.

cn (cell lysate with normal rabbit serum)

mn (medium with normal rabbit serum)

ca (cell lysate with rabbit anti-PC1 antiserum)

ma (medium with rabbit anti-PC1 antiserum)

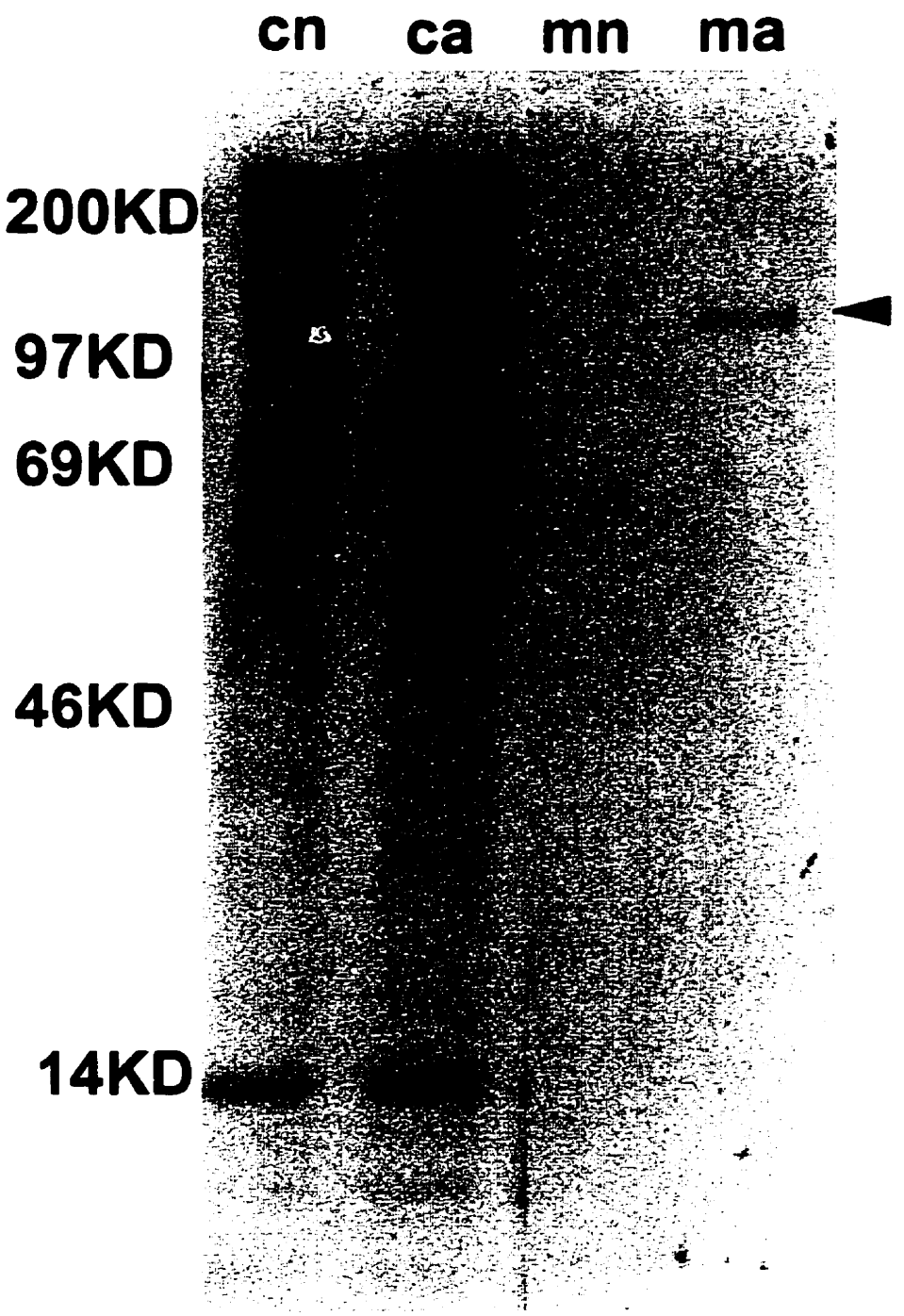

cn
ca
mn
ma
200KD
97KD
69KD
46KD
14KD

Figure 10. Immunoprecipitated protein of clone 6 on two dimensional electrophoresis gel.

Anti-PC1 immunoprecipitated proteins from clone 6 were resolved into four species with pIs between 5.58 and 6.17.

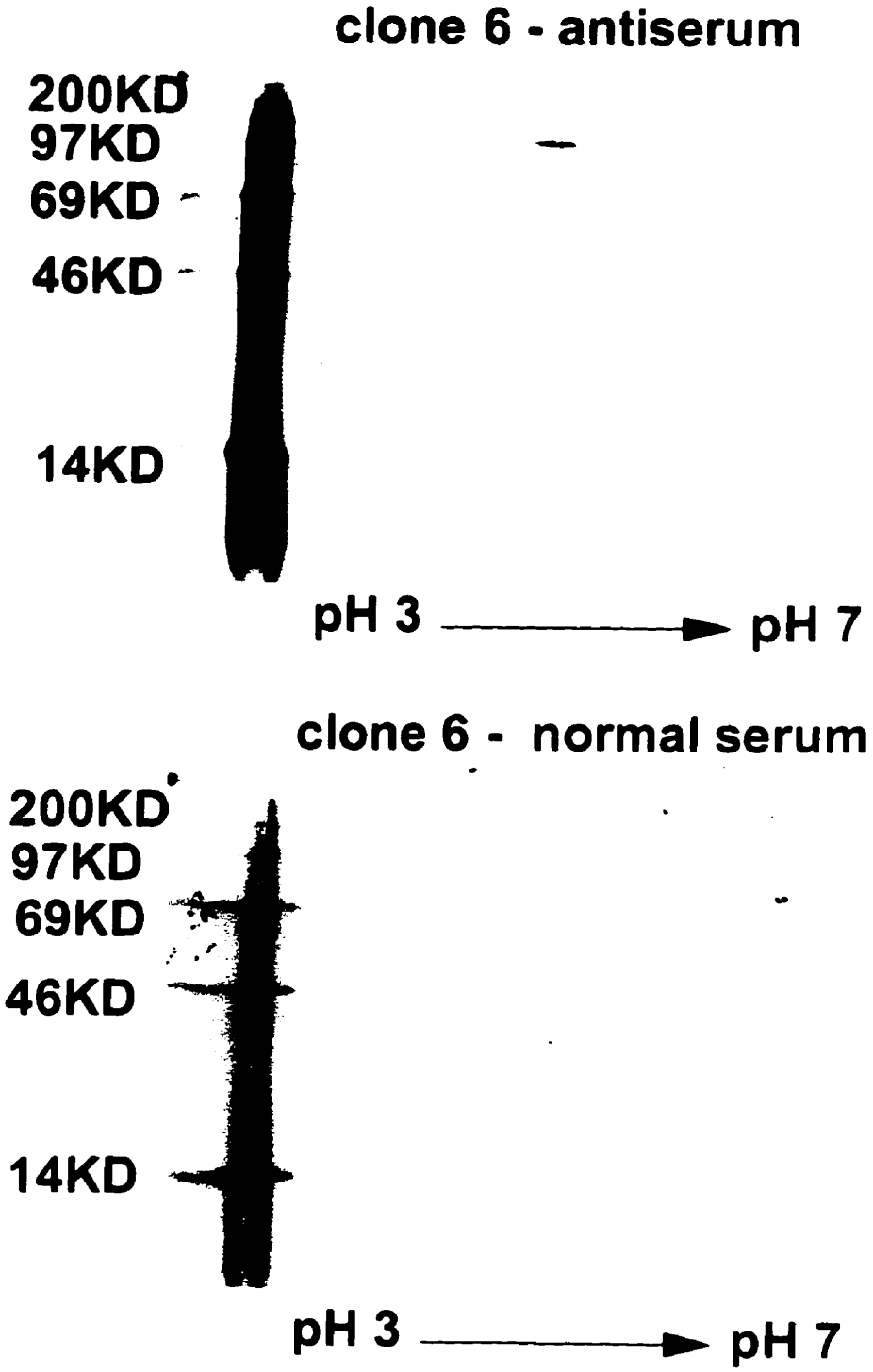

clone 6 - antiserum
200KD
97KD
69KD
46KD
14KD
pH 3
pH 7
clone 6 - normal serum
200KD
97KD
69KD
46KD
14KD
pH 3
pH 7

Figure 11. Comparison of cell lysate proteins patterns in 2D gel between clone 6 and clone-Neo.

Two-dimensional gel with a pI range from 3 to 7 and molecular weight range from 14 kDa to 220 kDa. The areas within the rectangles are enlarged and are shown in Fig. 12.

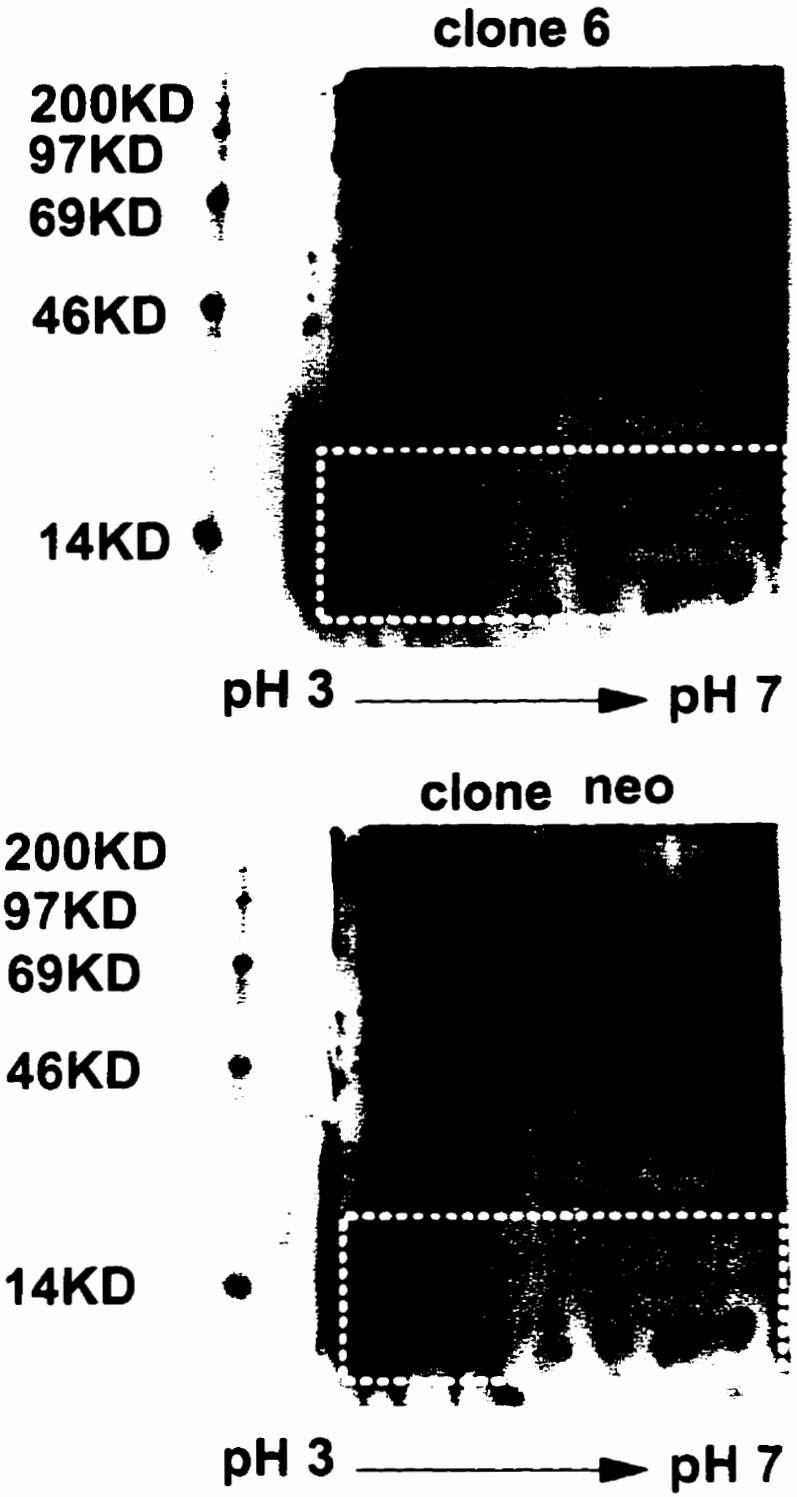
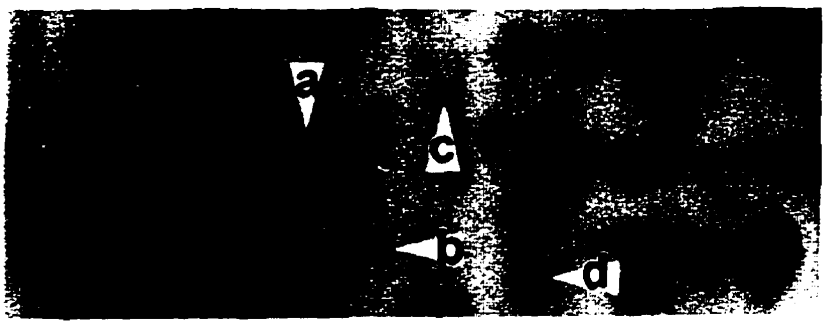

clone 6
200KD
97KD
69KD
46KD
14KD
pH 3
pH 7
clone neo
200KD
97KD
69KD
46KD
14KD
pH 3
pH 7

Figure 12. Comparison of four cell lysate proteins patterns in 2D gel between clone 6 and clone-Neo.

Clone 6 was compared with clone Neo, as a control, and observed differences were as follow proteins: a and b increased; c, decreased; d not changed.

clone 6



clone neo

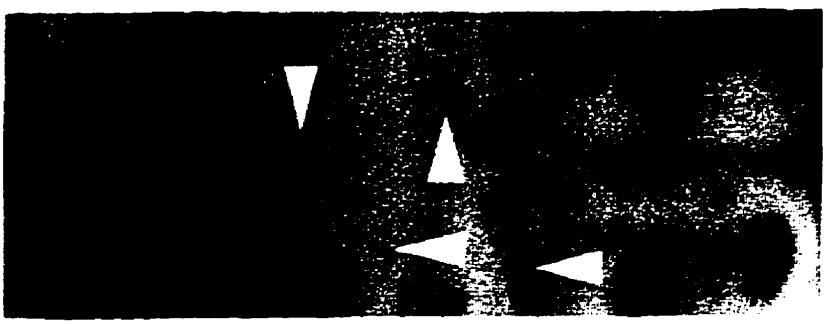
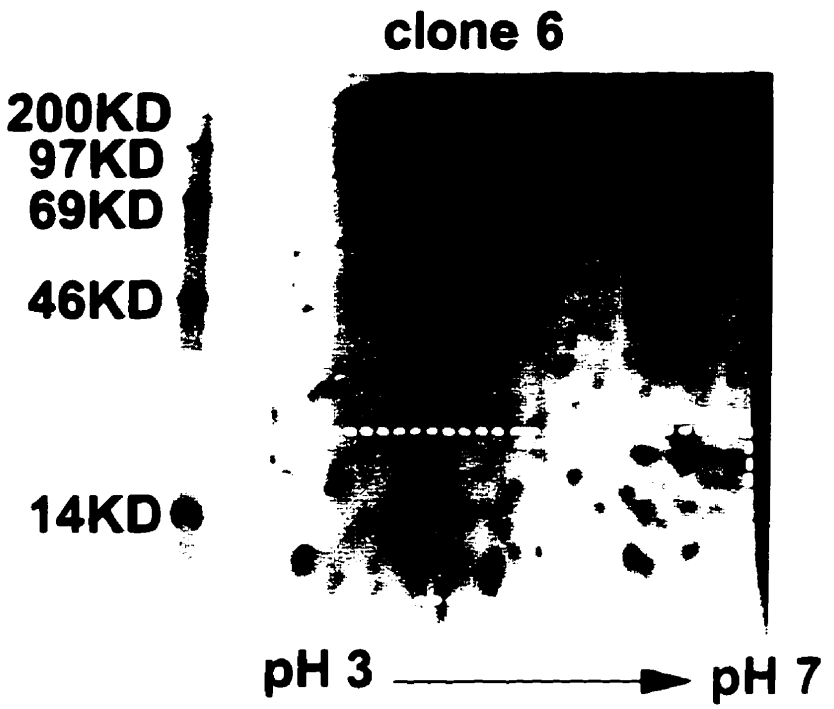


Figure 13. Comparison of cell lysate protein patterns in 2D gel between clone 6 and clone7, the first experiment.

Two-dimensional gel with a pI range from 3 to 7 and molecular weight range from 14 kDa to 220 kDa. The areas within the rectangles are enlarged and are shown in Fig. 14.


clone 6
200KD
97KD
69KD
46KD
14KD
pH 3
pH 7

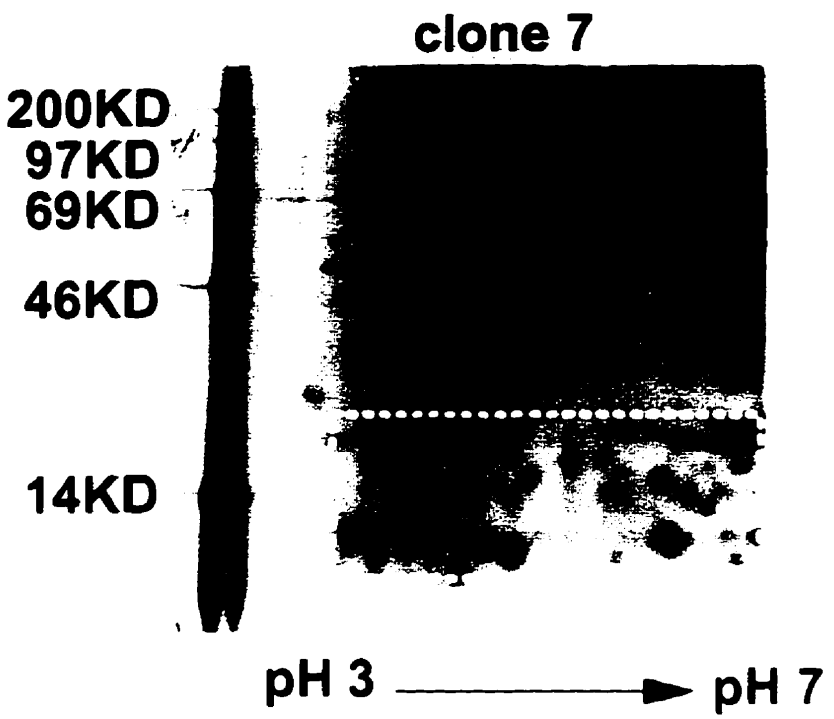
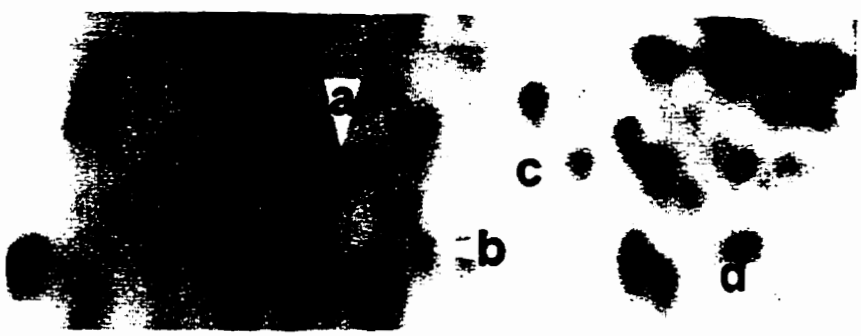

clone 7
200KD
97KD
69KD
46KD
14KD
pH 3
pH 7

Figure 14. Comparison of four cell lysate protein patterns in 2D gel between clone 6 and clone7, the first experiment.

Clone 6 was compared with clone 7, as a control, and observed differences in clone 6 were as follow proteins: a, b, and d increased; c, decreased.

clone 6



clone 7

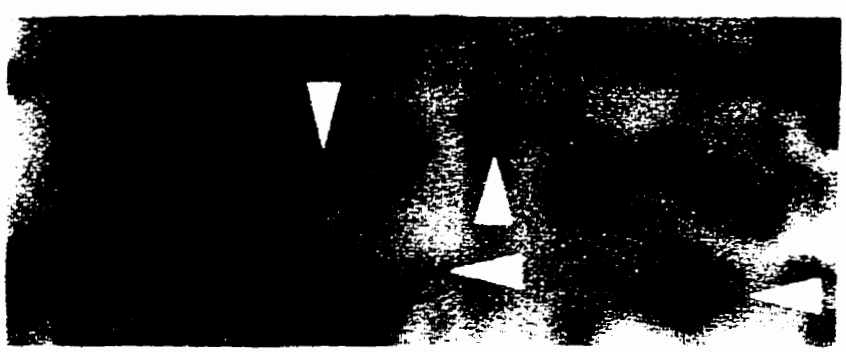
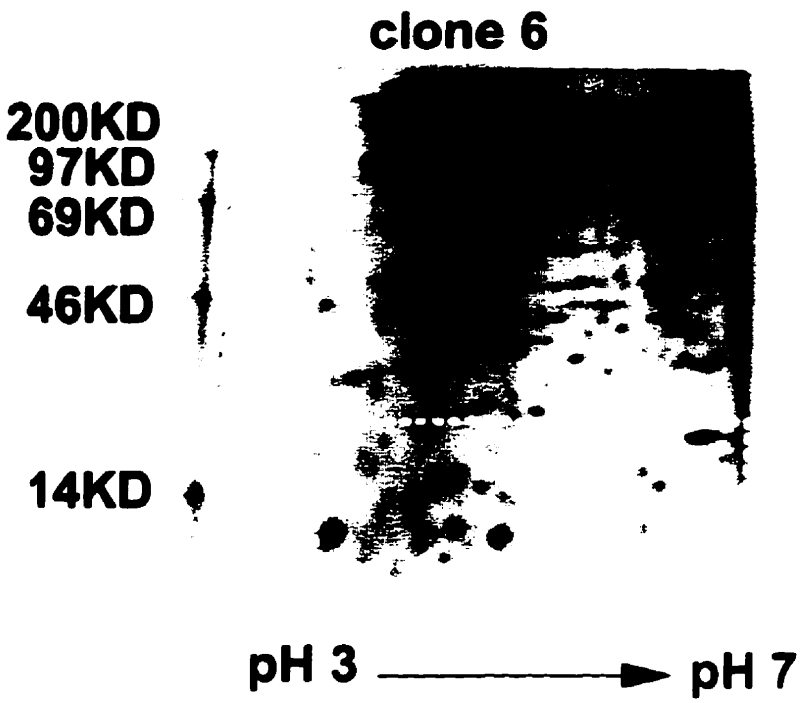


Figure 15. Comparison of cell lysate protein patterns in 2D gel between clone 6 and clone7, the second experiment.

Two-dimensional gel with a pI range from 3 to 7 and molecular weight range from 14 kDa to 220 kDa. The areas within the rectangles are enlarged and are shown in Fig. 16.


clone 6
200KD
97KD
69KD
46KD
14KD
pH 3
pH 7

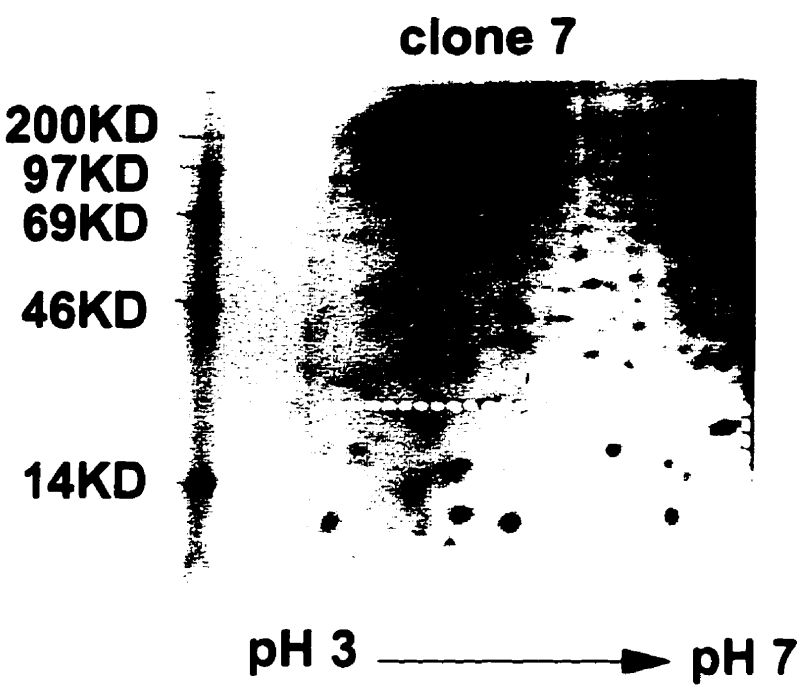
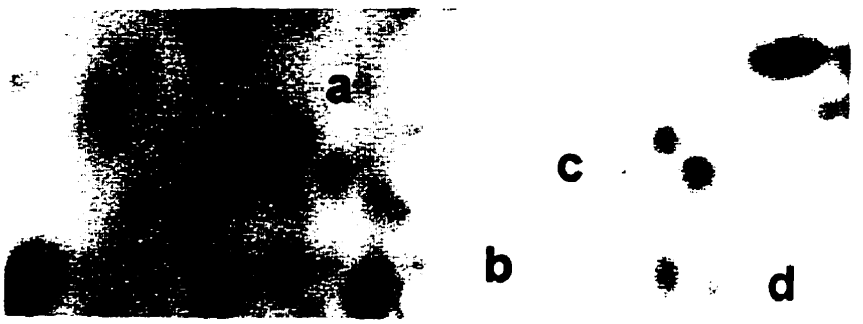

clone 7
200KD
97KD
69KD
46KD
14KD
pH 3
pH 7

Figure 16. Comparison of four cell lysate protein patterns in 2D gel between clone 6 and clone7, the second experiment.

Clone 6 was compared with clone 7, as a control, and observed differences were as follow

proteins: a, b, and d increased; c, decreased.

clone 6



clone 7

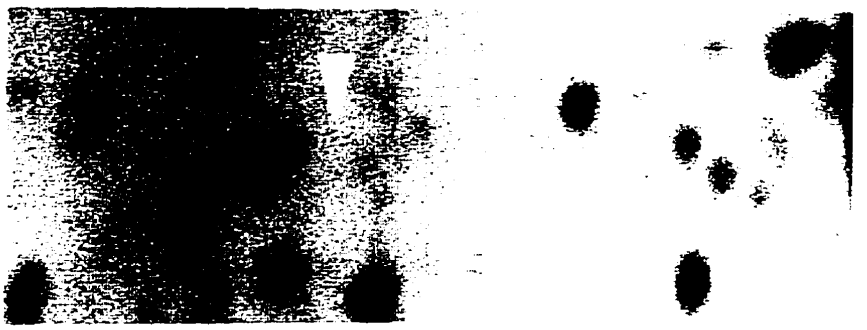
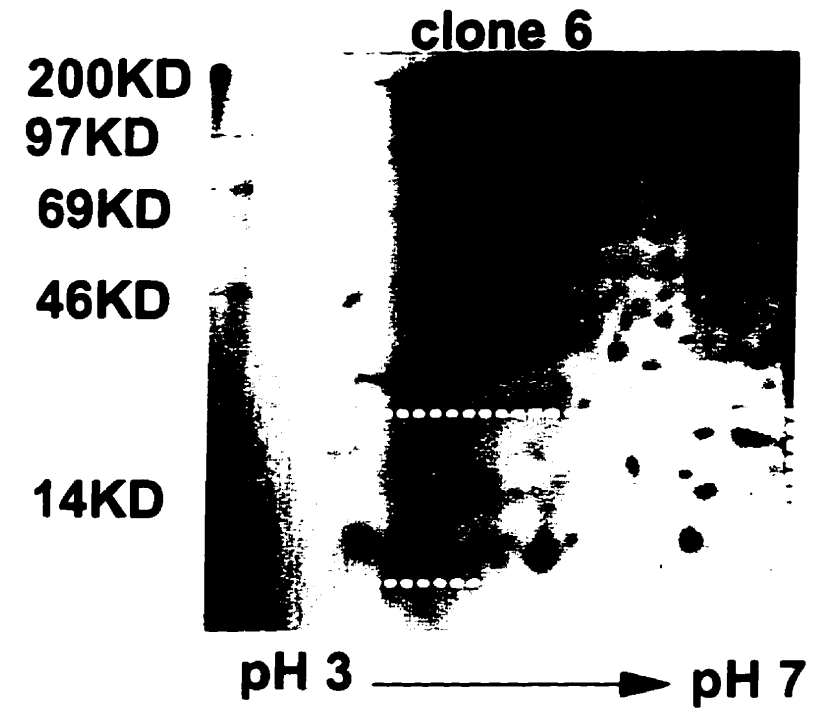


Figure 17. Comparison of cell lysate protein patterns in 2D gel between clone 6 and clone7, the third experiment.

Two-dimensional gel with a pI range from 3 to 7 and molecular weight range from 14 kDa to 220 kDa. The areas within the rectangles are enlarged and are shown in Fig. 18.


clone 6
200KD
97KD
69KD
46KD
14KD
pH 3
pH 7

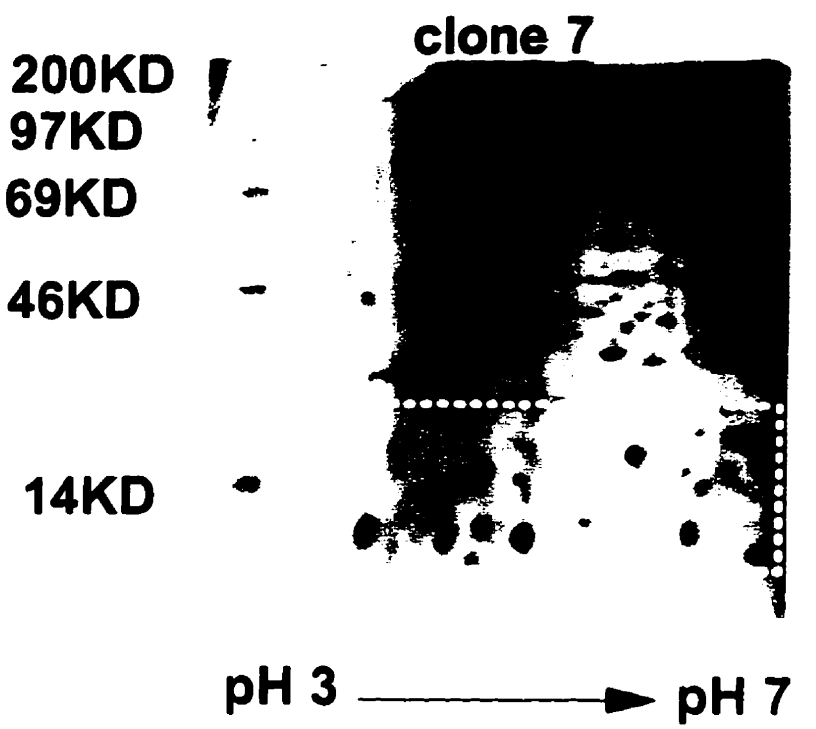

clone 7
200KD
97KD
69KD
46KD
14KD
pH 3
pH 7

Figure 18. Comparison of four cell lysate protein patterns in 2D gel between clone 6 and clone7, the third experiment.

Clone 6 was compared with clone 7, as a control, and observed differences were as follow

proteins: a and b decreased; c, increased; d, not changed.

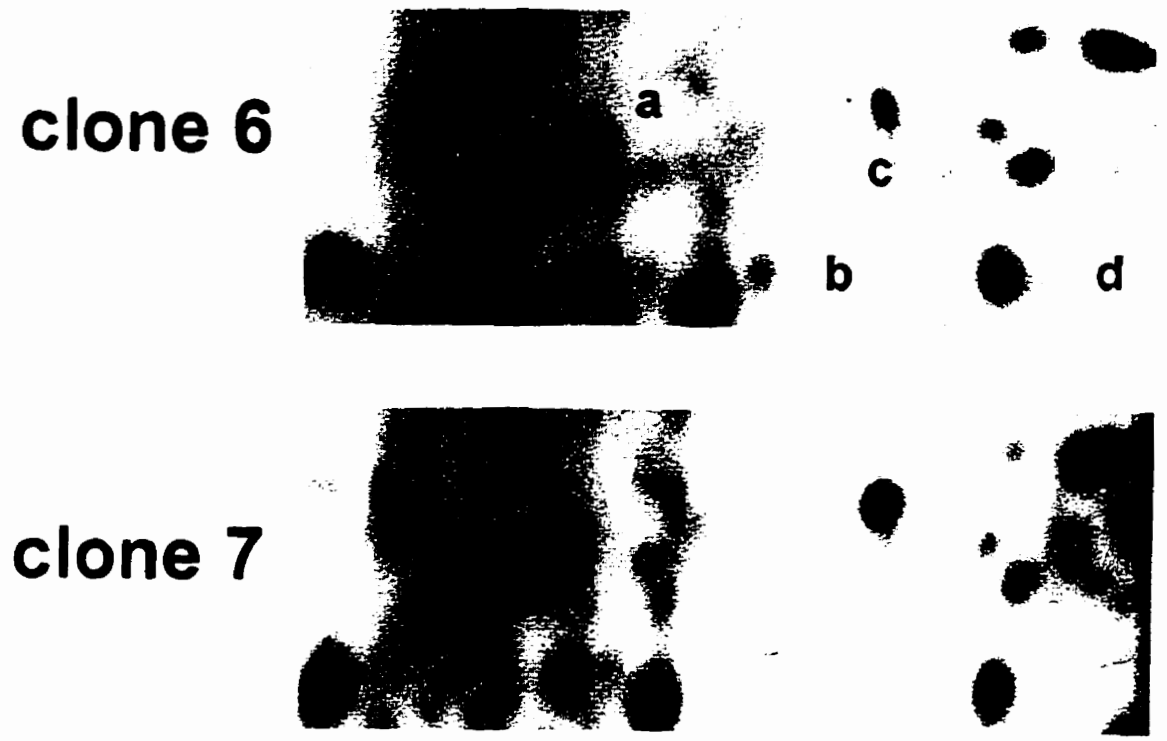
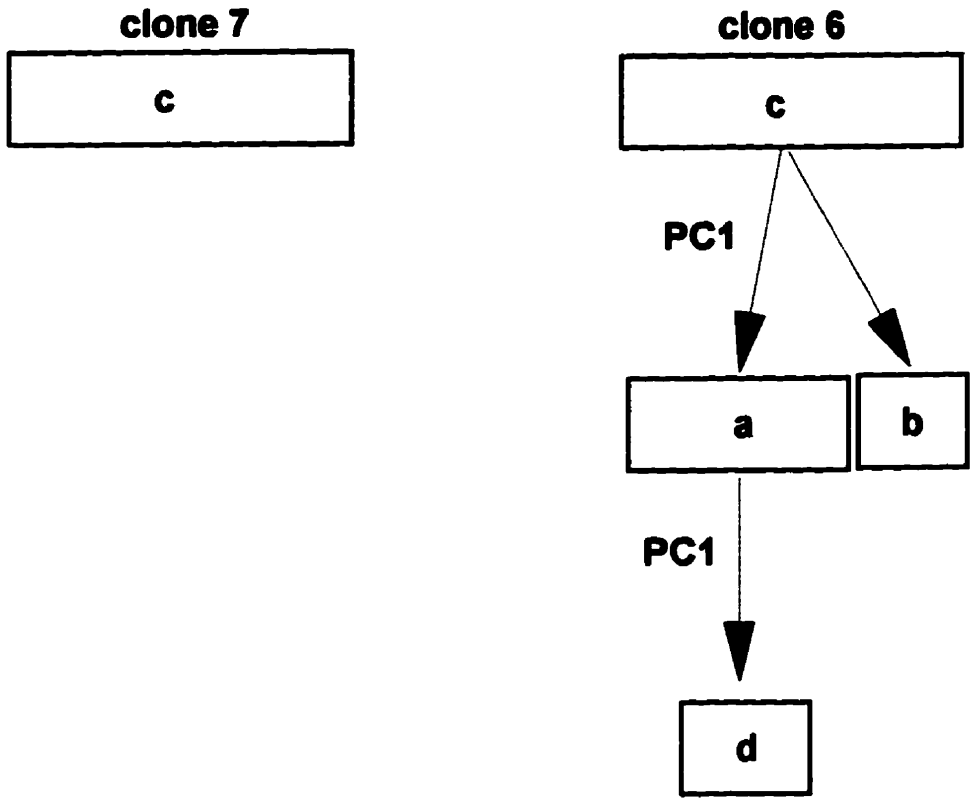

clone 6
a
c
b
d
clone 7

Figure 19. A hypothesis to explain new proteins in mPC1 transfectant clone 6 as detected in two dimensional gel.

Clone 6 was CMV-mPC1 stable transfected MCF-7 cell line, clone 7 was mock stable transfected MCF-7 cell line and served as a negative control. Chinges in four separate experiments, a, b, c, and d four reproducible protein spots on two-dimensional gels in clone 6 were observed. Protein a and b may be the products of PC1 target protein c, and protein d may be the product of PC1 target protein a.


clone 7
c
clone 6
c
PC1
a
b
PC1
d

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