Regulation of Cathepsin-D and pS2 Gene Expression by Growth Factors in MCF7 Human Breast Cancer Cells

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In MCF7 human breast cancer cells, cathepsin-D and pS2 mRNAs are specifically and directly induced by estrogens at the transcriptional level. We studied the regulation of expression of these two genes by growth factors that are also mitogenic in this cell line. We show that pS2 mRNA, like cathepsin-D mRNA, is rapidly induced 2- to 4-fold by epidermal growth factor. The effect of epidermal growth factor on these two mRNAs was dependent upon *de novo* protein synthesis, indicating a different mechanism of regulation than with estradiol. Other peptide growth factors, such as insulin, insulin-like growth factor I, and basic fibroblast growth factor, also increased up to 3-fold the steady state levels of the two mRNAs in MCF7 cells. The pS2 mRNA, but not cathepsin-D mRNA, was also induced up to 8-fold by protein kinase-C activation with 12-*O*-tetradecanoylphorbol-13-acetate, suggesting the possible involvement of this transduction pathway in pS2 mRNA induction. The effect of 12-*O*-tetradecanoylphorbol-13-acetate was time and dose dependent and required protein synthesis. In addition, treatment by agents elevating cAMP increased pS2 mRNA accumulation 4-fold, whereas it had no effect on cathepsin-D mRNA levels.

These results demonstrate that cathepsin-D and pS2 genes are under complex regulation in MCF7 cells, since growth factors stimulate their expression via indirect mechanisms contrasting with the primary transcriptional effects of estrogens. (Molecular Endocrinology 3: 552–558, 1989)

INTRODUCTION

The growth of metastatic human breast cancer cell lines containing estrogen receptors (MCF7, T47D, *etc.*) is stimulated by estrogens (1) and peptide growth factors such as epidermal growth factor (EGF), transforming growth factor-α or insulin-like growth factor I (IGF-I) (2, 3). Estrogens stimulate proliferation of these cells after the induction of several proteins (Ref. 4 and references therein). After activation of the nuclear estrogen receptor, the hormone-receptor complex directly regulates gene transcription after its binding to steroid-responsive elements located near or within the regulated gene.

Peptide growth factors also induce the expression of a few genes in the MCF7 cell line, by mechanisms that are still not understood. After the binding of growth factors to their specific receptors on the cell surface, signal transduction to the nucleus may involve either 1) directly, the growth factor-receptor complex itself, as in the case of steroid hormones, since growth factor receptors have been reported in the nucleus (5); or 2) more likely, activation of an intracellular pathway(s). Currently, two mechanisms of signal transduction are known to trigger effects of growth factors: coupling through activation of the protein tyrosine kinase activity of the receptors or coupling through activation of Ca^{2+}- and phospholipid-dependent protein kinase-C after receptor-mediated hydrolysis of inositol phospholipids (6).

Two genes, coding for the secreted pS2 protein (7) and cathepsin-D (8), have recently been shown to be transcriptionally and directly regulated by estrogens in MCF7 cells. The pS2 gene encodes a small protein (9) similar in sequence to a porcine pancreatic protein shown to inhibit gastrointestinal mobility and gastric secretion (10). The pS2 protein is specifically expressed and secreted by a subclass of estrogen receptor-positive breast cancer cells (11) and also by mucosa cells of normal stomach (10). In human breast cancer cells the lysosomal protease cathepsin-D is also regulated by estrogens and secreted in its proenzyme form (12–14). This procathepsin-D, which we first described as a 52,000 mol wt protein, was found to be mitogenic *in vitro* (15) and capable of degrading the extracellular matrix (16), suggesting a potential role in mammary carcinogenesis (17).

In the present study we examined the effects of growth factors on pS2 and cathepsin-D mRNA accumulation in MCF7 cells and found that the two genes are regulated by estrogens and growth factors through separate mechanisms.

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RESULTS

Growth Factors Induce Cathepsin-D and pS2 mRNAs

We evaluated the effect of EGF on the levels of pS2 and cathepsin-D mRNAs in MCF7 cells deprived of estrogens. Figure 1 shows a 4-fold induction of pS2 mRNA by EGF, which rapidly reached a maximum at 6 h, but was transitory, since it was not observed after 2 days of treatment. By contrast, the induction of cathepsin-D mRNA previously described (8) was not as great, but was more prolonged, suggesting a different mechanism of induction or different stability of the mRNA under these conditions.

The effects of other growth factors that stimulate MCF7 cell growth via specific receptors on cathepsin-D and pS2 mRNAs were also evaluated. As shown in Fig. 2, insulin, IGF-I, and basic fibroblast growth factor (FGF) treatments also increased both cathepsin-D and pS2 mRNA accumulation. After scanning autoradiographs of different exposures, the degrees of stimulation were estimated to be 2- to 3-fold for cathepsin-D and pS2 mRNAs. However, in all experiments estradiol was more efficient than growth factors in stimulating accumulation of these mRNAs.

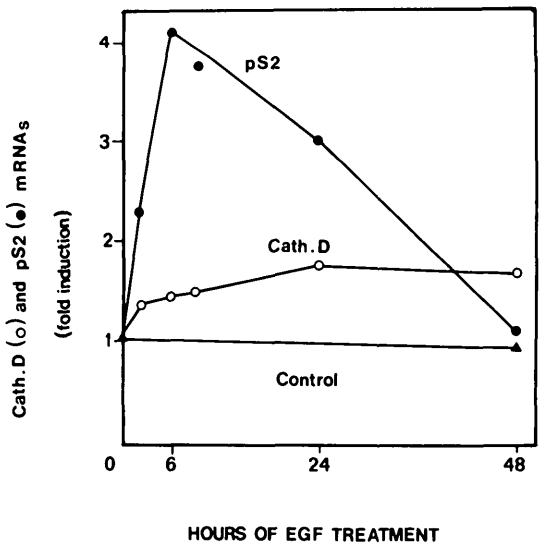

Fig. 1. Time Course of Cathepsin-D and pS2 mRNA Induction by EGF

MCF7 cells were cultured in medium containing charcoal-stripped serum without insulin and then for the indicated times in the presence of 4 nM EGF (● and ○) or with solvent alone (control; ▲). Total RNA (20 μg) was analyzed by Northern blotting as described in *Materials and Methods*. The levels of pS2 cDNA hybridized to the 0.6-kilobase pS2 mRNA were determined by densitometric scanning of autoradiographs. Results were corrected according to the constant 36B4 mRNA and are expressed relative to the control value. The time course of cathepsin-D (Cath-D) mRNA induction by EGF, previously reported (8), is shown for comparison.

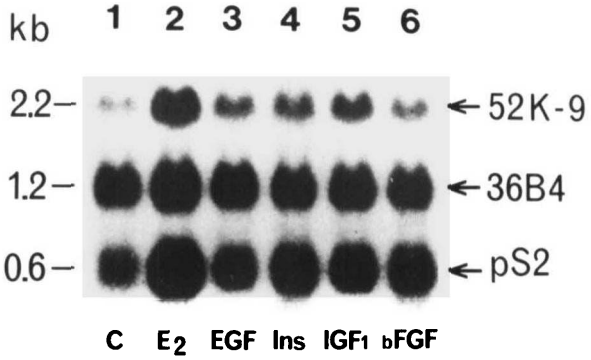

Fig. 2. Effects of Growth Factors on Cathepsin-D and pS2 mRNA Levels in MCF7 Cells

MCF7 cells were grown in stripped medium for 3 days and then in serum-free medium for 1 day as described in *Materials and Methods*. Cells were then stimulated for 24 h with 10 nM estradiol (E_2; lane 2), 4 nM EGF (lane 3), 100 nM insulin (Ins; lane 4), 5 nM IGF-I (lane 5), or 0.6 nM basic FGF (lane 6) or not treated [control (C); lane 1]. Total RNA was analyzed as described in *Materials and Methods*. Filters were hybridized with 52K-9 cDNA (14) to probe cathepsin-D mRNA and with pS2 cDNA. The 36B4 probe, which detects a constant RNA species, was used as a control. The lengths of the RNA species detected on the autoradiograph are indicated in kilobases (kb).

Regulation by Growth Factors and Estrogens Involves Different Mechanisms

We then tested whether the induction of cathepsin-D and pS2 mRNAs by growth factors was dependent on *de novo* protein synthesis, using cycloheximide at concentrations that block protein synthesis by 96% (not shown). The induction of cathepsin-D and pS2 mRNAs by EGF or IGF-I was drastically decreased by cycloheximide treatment (Fig. 3). These results suggest that EGF and IGF-I, unlike estradiol, increase levels of the two mRNAs by acting through a mechanism involving synthesis of a protein intermediate.

The additivity of estradiol and EGF stimulations of cathepsin-D and pS2 mRNA accumulation is shown in Fig. 4. Enhancement of mRNA accumulation by simultaneous treatment with the two inducers corresponded to the sum of inductions obtained by separate treatments (96% and 104% for cathepsin-D and pS2 mRNAs, respectively). However, the additive effect of estradiol on the optimal EGF-mediated induction was more evident, since estradiol is a stronger inducer than EGF. Moreover, this additivity was demonstrated at the protein level by immunometric assay of the secreted procathepsin-D (data not shown).

These results indicate that the regulations of these two genes by EGF and estradiol involve distinct mechanisms, since EGF induction required *de novo* protein synthesis and was independent of estrogen receptor modulation by estradiol or antiestrogens.

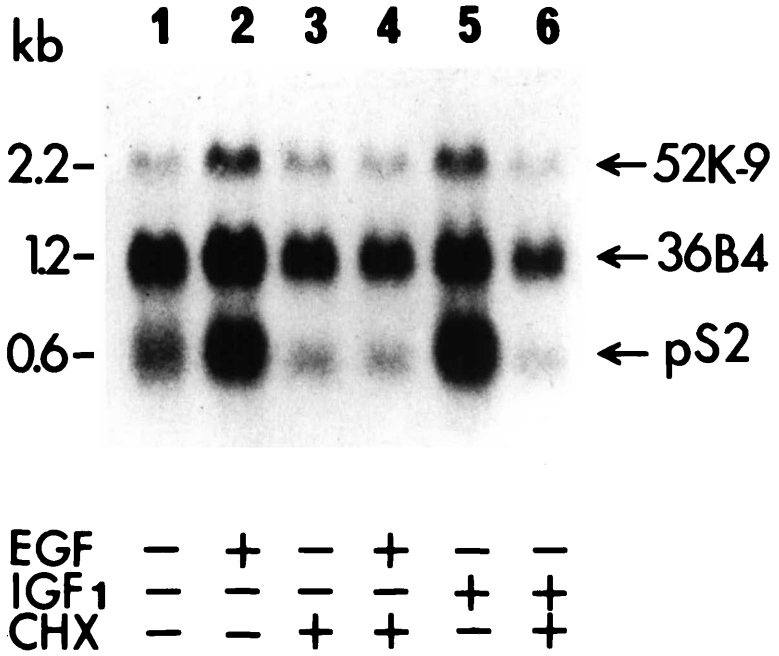

Fig. 3. Inhibition of Protein Synthesis Prevents Induction of Cathepsin-D and pS2 mRNA by EGF and IGF-I

MCF7 cells grown in stripped medium were not treated or pretreated for 1 h with 50 μM cycloheximide (CHX) and then with 4 nM EGF, 5 nM IGF-I, or solvent alone for 14 h, with cycloheximide still present. Total RNA was analyzed by Northern blot as described in Fig. 2. kb, Kilobases.

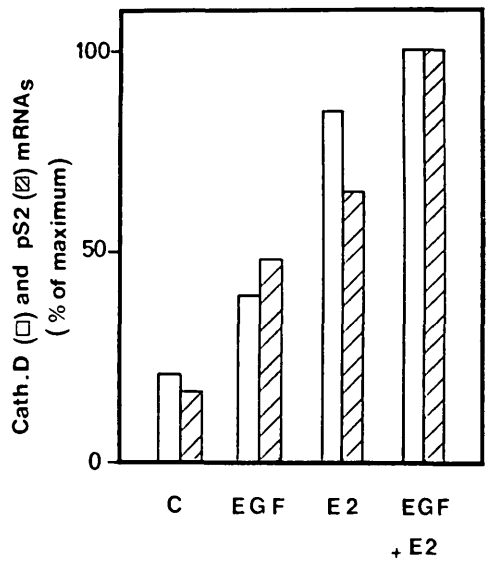

Fig. 4. Effects of Estradiol on Cathepsin-D and pS2 mRNA Induction by EGF in MCF7 Cells

MCF7 cells stripped of steroids were treated for 24 h with solvent alone (C), 8 nM EGF, 1 nM estradiol (E2), or 8 nM EGF plus 1 nM estradiol (EGF + E2). Total RNA was assayed for cathepsin-D (Cath.D) and pS2 mRNA as described in *Materials and Methods*. Results obtained from densitometric scanning of autoradiographs were corrected for slight variations in the amount of RNA loaded in each track and are expressed as percentages of the maximum value.

Effect of Protein Kinase-C Activation on the Expression of Cathepsin-D and pS2 Genes

The inductions of cathepsin-D and pS2 mRNA by growth factors may be triggered by the intrinsic tyrosine kinase activity of these growth factor receptors or by other protein kinases, such as the Ca^{2+}- and phospholipid-dependent protein kinase-C.

To test the second hypothesis we used 12-*O*-tetradecanoylphorbol-13-acetate (TPA), which directly stimulates protein kinase-C activity and mimics some effects obtained after activation of the inositol lipid cycle. Treatment of MCF7 cells with 100 nM TPA increased pS2 mRNA in a time-dependent manner, reaching 8-fold stimulation after 24 h (Fig. 5). By contrast, cathepsin-D mRNA was weakly affected by TPA, reaching a less than 2-fold increase at 24 h. The effect of TPA on pS2 mRNA was strongly reduced when cells were pretreated with cycloheximide, suggesting that TPA, like EGF and IGF-I, was acting via the synthesis of a protein intermediate (Fig. 6). Moreover, the induction of pS2 mRNA by 100 nM TPA was totally abolished by actinomycin-D (3 μg/ml), indicating that a transcriptional step is involved (not shown). These results suggest that the signal transduction pathway involving protein kinase-C activation (by Ca^{2+} and diacylglycerol) is probably not involved in the mechanism of cathepsin-D mRNA induction by growth factors, but may be responsible for that of pS2 mRNA.

Effect of cAMP and Ca^{2+}

Another secondary messenger of peptide hormones, cAMP, was also tested on the induction of cathepsin-D

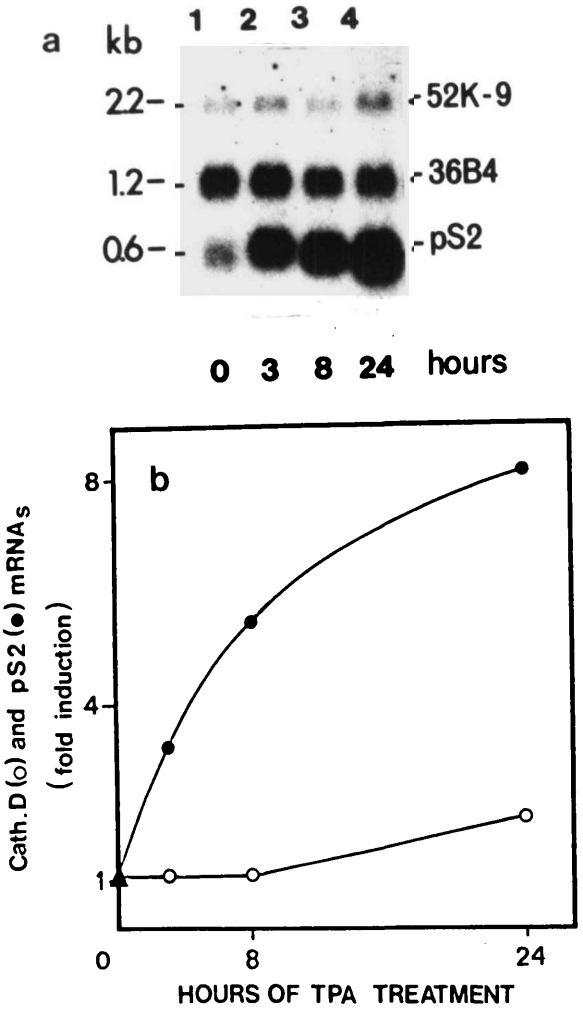

Fig. 5. Dissociated Effect of Phorbol Ester (TPA) on the Accumulation of Cathepsin-D and pS2 mRNAs

MCF7 cells were stripped of steroids and then cultured for the indicated times with 100 nM TPA. Control cells (▲) received dimethylsulfoxide alone for 24 h. Total RNA was analyzed as described in Fig. 2. Hybridization was done with 52K-9 cDNA to probe cathepsin-D (Cath.D) mRNA and with 36B4 and pS2 cDNAs. a, Autoradiograph of Northern blots. b, Densitometric scanning of pS2 and cathepsin-D mRNA levels corrected for slight variations in the levels of constant 36B4 RNA. kb, Kilobases.

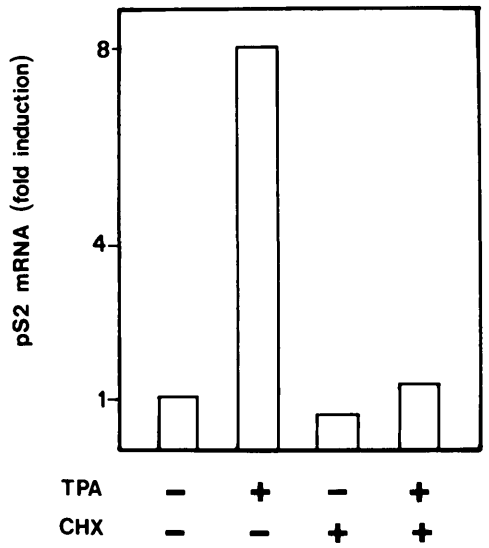

Fig. 6. Effect of Cycloheximide on pS2 mRNA Induction by TPA in MCF7 Cells

Steroid-stripped MCF7 cells were not treated or pretreated with 50 μM cycloheximide (CHX) for 1 h and then cultured for 24 h in the presence of 100 nM TPA or solvent alone. Total RNA was extracted from MCF7 cells, and pS2 mRNA was assayed as described in Fig. 2. Results were corrected according to the 36B4 level.

and pS2 mRNAs in MCF7 cells. The intracellular concentration of cAMP was increased by simultaneous treatment of MCF7 cells with cholera toxin, which activates adenylate cyclase, and with 3-isobutyl-1-methylxanthine (IBMX), which inhibits phosphodiesterase activity. As shown in Fig. 7, this treatment resulted in a 4-fold increase in pS2 mRNA accumulation after 24 h, whereas cathepsin-D mRNA was not affected (lanes 1 and 2). This effect was confirmed using 2.5 mM 8-bromo-cAMP, which increased pS2 mRNA 2-fold between 2 and 24 h of treatment (not shown).

The possible role of Ca^{2+} was studied using the calcium ionophore A23187. A23187 alone (lanes 1 and 3) had no effect on the expression of either gene and did not affect the induction of pS2 mRNA by cAMP (lanes 2 and 4). We confirmed that under these conditions A23187 increased the calcium influx rate (375% of that in unstimulated cells), determined by measuring the initial uptake of $^{45}CaCl_2$ in MCF7 cells.

DISCUSSION

The regulation of cathepsin-D and pS2 mRNAs by growth factors was studied in MCF7 human breast cancer cells. We demonstrate that both of these genes, which are transcriptionally and directly regulated by estrogens in MCF7 cells, are also induced by several peptide growth factors (EGF, insulin, IGF-I, and basic FGF), indicating that their expression is associated with the mitogenic activity of mammary cancer cells. However, these peptide mitogens were less active than estradiol in increasing the steady state levels of the two mRNAs. Several findings indicate that the estradiol and growth factor stimulations involve separate mechanisms. The two inductions are additive, and unlike estrogens, the action of growth factors is prevented by protein synthesis inhibition. Our results also suggest that the mechanism of estradiol action on these genes is more complex than initially described. It has been demonstrated that estrogens increase the secretion of several growth factors in MCF7 cells, such as transforming growth factor-α and IGF-I (18). On the basis of our results, it would appear that this increased secretion of growth factors then indirectly increases the expression of cathepsin-D and pS2 genes. However, the early

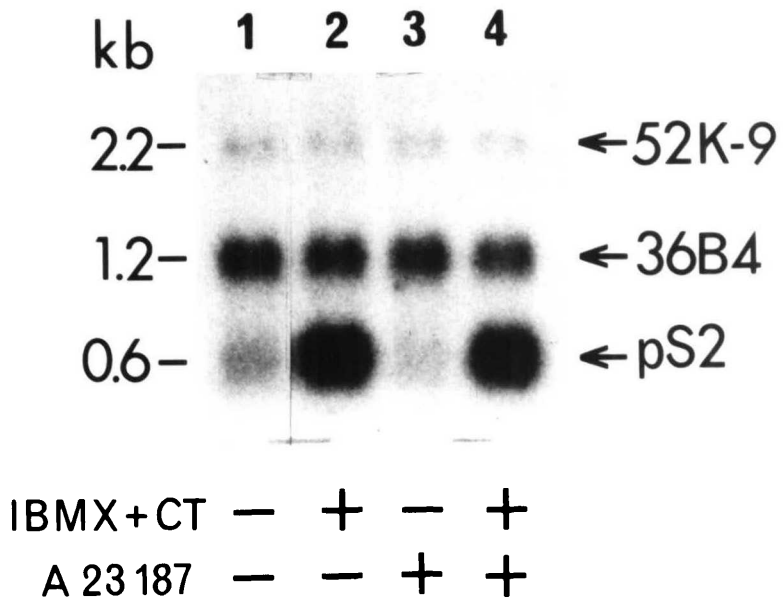

Fig. 7. Effect of Cellular Concentrations of cAMP and Ca^{2+} on Cathepsin-D and pS2 mRNA Levels

MCF7 cells were stripped of steroids and then stimulated for 16 h with 1 μg/ml cholera toxin (CT) plus 0.1 mM IBMX (lane 2), 0.5 μM A23187 (lane 3), cholera toxin plus IBMX and A23187 (lane 4), or solvent alone (lane 1). Total RNA was analyzed and hybridized as described in Fig. 2, and the autoradiograph is shown. The length of the three mRNA species is indicated in kilobases (kb).

stimulation of gene expression by estradiol is mediated via estrogen receptors and not via growth factors, since it is insensitive to protein synthesis inhibition. Therefore, it appears that a common set of genes, including c-*fos*, c-*myc* (19), and those of cathepsin-D and pS2, is regulated at the mRNA level by both estradiol and growth factors in MCF7 cells. The regulation of these genes by estradiol and growth factors suggests that the tissue concentration of these markers may be correlated with the degree of proliferation of breast cancers, as indicated by immunohistochemical studies of 52K cathepsin-D in benign mastopathies (20).

Treatment of MCF7 cells with phorbol ester or factors that increase intracellular cAMP also significantly increased pS2 mRNA accumulation, whereas the cathepsin-D mRNA level was only slightly affected if at all. These results suggest that in addition to the intrinsic tyrosine kinase activity of growth factor receptors, other transduction pathways involving activation of Ca^{2+}- and phospholipid-dependent protein kinase-C or cAMP-dependent protein kinases may be involved in pS2 mRNA induction.

We also show that cathepsin-D and pS2 mRNA induction by EGF or IGF-I is prevented by cycloheximide treatment. It has been demonstrated that *de novo* protein synthesis is also required for induction of the transin gene by EGF (21), whereas EGF-mediated increases in c-*fos* (22) and PRL (23) transcription can occur in the absence of ongoing protein synthesis. Further experiments are needed to determine the level of regulation by growth factors, which may involve mRNA stabilization (24) or activation of transcription (21–23). In most cases, growth factors stimulate gene transcription after the binding of *trans*-acting factors to specific genomic sequences. Such responsive elements have previously been described in several genes, including c-*fos* (25) and PRL (26). In another group of genes regulated by TPA, including those of collagenase, metallothioneine II, and simian virus-40, the *trans*-acting factor AP-1 has been characterized (27) and is related to the product of the proto-oncogene c-*jun* (28).

It has been recently shown, by transfection experiments, that the pS2 5′ flanking region behaves as an orientation-independent estrogen-responsive element (29–31). A similar approach using 5′ regions of pS2 and cathepsin-D genes should make it possible to demonstrate whether growth factors and TPA also act at the transcriptional level and should allow characterization of the genomic sequences and *trans*-acting factors involved.

MATERIALS AND METHODS

Materials

EGF and insulin were purchased from Collaborative Research, Inc. (Waltham, MA), and IGF-I from Amersham (Arlington Heights, IL). Basic FGF was a gift from D. Barritault (Université Paris Val-de-Marne, Créteil, France). TPA, cycloheximide, cholera toxin, IBMX, and A23187 were obtained from Sigma (St. Louis, MO).

Cell Culture

MCF7 human breast cancer cells were obtained from the Michigan Cancer Foundation and routinely maintained in Dulbecco's Modified Eagle's Medium supplemented with 10%

fetal calf serum. For experiments, cells were grown for at least 9 days in phenol red-free medium containing 10% fetal calf serum treated with dextran-coated charcoal. Medium was changed every 2 days after washes with PBS. In an attempt to decrease exogenous growth factors, the withdrawal procedure of the experiment shown in Fig. 2 was modified. Cells were cultured for 3 days in medium containing charcoal-stripped serum and then in serum-free medium supplemented with transferrin (10 μg/ml), sodium selenite (30 nM), and 0.2% BSA for 1 day. All stimulations were performed in medium containing 10% charcoal-treated serum for the indicated times, while control cells received solvent alone. Cells were then harvested by trypsinization, and after washes and centrifugation, the pellet was frozen in liquid nitrogen and stored at −80 C until RNA preparation.

RNA Extraction and Northern Blot Analysis

Total RNA was extracted by the method of Auffray and Rougeon (32), electrophoresed on a 1% agarose formaldehyde-denaturing gel, and transferred to nitrocellulose. Double stranded 36B4 and pS2 cDNA (33) (gifts from P. Chambon, Faculté de Médecine, Strasbourg, France) or 52K-9 cDNA, which corresponds to most of the coding sequence of cathepsin-D mRNA (14), were ^{32}P labeled in the vector using random primers. Hybridization and washes were performed as previously described (8). Autoradiographs were densitometrically scanned to determine cathepsin-D and pS2 mRNA levels, which were corrected for slight variations in the amount of RNA loaded on each track using 36B4 cDNA.

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