Hormonal Regulation of Synthesis and Secretion of pS2 Protein Relevant to Growth of Human Breast Cancer Cells (MCF-7)

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ABSTRACT

We have recently identified human epidermal growth factor-like immunoreactive factor synthesized and secreted by human breast cancer cells (MCF-7) as a secretory protein encoded by the pS2 gene, the transcription of which is directly induced by estrogen. We demonstrated in this paper that synthesis and secretion of pS2 protein as well as pS2 mRNA were induced about 5-fold specifically by physiological concentrations of estrogen, which stimulated growth of the cells about 5-fold. Stimulative effects of estrogen on both cell growth and synthesis/secretion of pS2 protein were inhibited completely by actinomycin D, cycloheximide, and antiestrogen. However, the increase in DNA synthesis from 6 h after the start of treatment of the cells with estrogen preceded the increase in the amount of pS2 protein in the culture medium from 12 h after that. Furthermore purified pS2 protein did not stimulate DNA synthesis of the cells. These results suggest that induction of pS2 protein by estrogen is not involved in the growth-stimulating effect of estrogen in MCF-7 cells.

INTRODUCTION

Tumor cells have been shown to acquire relatively autonomous growth by responding to polypeptide growth factors produced by their own cells, the mechanisms being termed an autocrine system (1). Since the discovery of a close similarity between v-sis oncogene product and B chain of platelet-derived growth factor, direct relationships between oncogene products and cellular growth factors or their receptors have been established (2).

These tight linkages were further expanded to include hormone receptors, for c-erb A was shown to encode thyroid hormone receptor (3, 4) and various steroid hormone receptors, i.e., estrogen, glucocorticoid, and progesterone receptors are also related to the c-erb A oncogene product (5). Thus aberrant transduction mechanisms by way of growth factors (hormones) and their receptor pathway are thought to have a central role in malignant transformation.

In order to obtain more information about the relationship between EGF and malignant transformation, we developed a sensitive enzyme immunoassay system for human EGF (6) and found that certain cancer cells, i.e., human breast cancer cells (MCF-7) and human gastric cancer cells (MKN-45 and KATO-III), synthesized and secreted a large amount of human EGF-like immunoreactive factor (designated EGF-LI) into the culture medium (7-10). To investigate the role of EGF-LI in cancer cells, we recently isolated EGF-LI in pure form from serum-free medium conditioned by MCF-7 cells and identified it as pS2 protein by NH2-terminal sequence analysis (11). pS2 protein is encoded by a gene the transcription of which is directly induced by estrogen, one of the growth-stimulating agents toward MCF-7 cells (12). The fact that pS2 protein is a cysteine-rich low molecular weight polypeptide produced and secreted by cancer cells evoked the possibility that it is an estrogen-inducible growth factor. To assess this possibility, we investigated herein the effects of various hormones, especially of estrogen, on synthesis and secretion of pS2 protein relevant to the growth of MCF-7 cells.

MATERIALS AND METHODS

Materials. β-estradiol, testosterone propionate, and progesterone were obtained from Tokyo Kasei (Japan); hydrocortisone, tamoxifen, and nafodixine were from Sigma; actinomycin D was from Makor; and cycloheximide was from Nakarai Chemicals (Japan). pS2 protein and human EGF were purified as described previously (11, 13).

Cell Culture Procedures. MCF-7 cells (American Type Culture Collection HTB22) were cultured in a humidified atmosphere of 5% CO2/95% air at 37°C in MEM containing nonessential amino acids (0.1 mm), sodium pyruvate (0.1 mm), 10% FCS (Bocknek), and kanamycin (60 μg/ml), hereafter referred to MEM-FCS. When the cells reached the confluent state, they were seeded into 24-well plates at a density of 4 x 104 cells/well and cultured for 2 days in MEM-FCS to spread sparsely. Then the medium was replaced with that containing 0.5% BSA (fraction V; Armour) instead of 10% FCS (MEM-FBSA) to avoid the effects of serum components and endogenous steroid hormones present in FCS (14). After a 24-h incubation in MEM-FBSA, the cells were cultured in fresh MEM-FBSA containing the desired hormones or inhibitors for the indicated time. In all experiments, the medium contained phenol red which acts as a weak estrogen (15).

BALB/c 3T3 cells were cultured in Dulbecco’s modified MEM containing 5% FCS, penicillin (50 units/ml), and streptomycin (0.05 mg/ml). When the cells reached the confluent state, they were seeded into 24-well plates at a density of 2 x 104 cells/well and cultured for 2 days to reach the subconfluent state. Then the medium was replaced with Dulbecco’s modified MEM containing 0.5% FCS. After a 48-h incubation, pS2 protein or human EGF was added to the culture medium.

Assay of pS2 Protein. Assay of pS2 protein (EGF-LI) samples was performed using the two-site enzyme immunoassay for human EGF as described previously (6, 7). The limit of detection of pS2 protein (EGF-LI) was as low as 1 pg/assay tube. This assay system does not detect other growth factors, such as human transforming growth factor type α (10; and Footnote 4).

Protein Determination. Protein was determined by the method of Lowry et al. (16) with some modifications. Bovine serum albumin was used as a standard.

Determination of DNA Synthesis. Tritiated [methyl-3H]thymidine (6.9 Ci/mmol; ICN Radiochemicals), 1 μCi/well, was included in the culture medium during the last 1 h of the incubation period. The medium was removed and the cells were dissolved in 1 ml of 0.5% sodium lauryl sulfate. The acid-insoluble materials precipitated with 100 μl of 100% trichloroacetic acid were collected on glass microfiber filters (Whatman GF/C). After extensive washing of the precipitates with 5% trichloroacetic acid and 95% ethanol, the 3H radioactivity on the filter was determined in a liquid scintillation counter.

* Our unpublished data.
RESULTS

Effects of Various Hormones on Growth of MCF-7 Cells and Synthesis/Secretion of pS2 Protein. Fig. 1 shows dose titration of the effects of various steroid hormones, i.e., estrogen, androgen, and progesterone, on DNA synthesis of MCF-7 cells and the amount of pS2 protein in the medium. β-Estradiol stimulated [3H]thymidine incorporation into DNA 5-fold and correspondingly increased cell protein content 2-fold at concentrations of 10^{-11} - 10^{-6} M (Fig. 1A) as reported (17). At the same time, the amount of pS2 protein in the medium was increased 5-fold. The concentration of β-estradiol for half-maximal effects on cell growth and induction of pS2 protein were less than 10^{-11} M. Testosterone stimulated DNA synthesis and increased cell protein content to an extent almost comparable to that by β-estradiol (Fig. 1B) as reported (18). Although the amount of pS2 protein in the medium was increased slightly by adding high concentrations of testosterone, the extent of induction was much less than that by β-estradiol. On the other hand, progesterone neither stimulated cell growth nor induced pS2 protein (Fig. 1C). Hydrocortisone also did not induce pS2 protein (data not shown). We already showed that insulin had no effect on induction of pS2 protein although it stimulated growth of the cells (10).

From these results we conclude that synthesis and secretion of pS2 protein are induced specifically by physiological concentrations of β-estradiol, which show growth-stimulating activity toward MCF-7 cells.

Effects of Various Inhibitors on Growth Stimulation and Induction of pS2 Protein by β-Estradiol. To examine whether induction of pS2 protein by β-estradiol is mediated by estrogen receptor, the effects of antiestrogens such as tamoxifen and nafoxidine were studied. Tamoxifen or nafoxidine alone did not alter the amount of pS2 protein in the medium significantly (data not shown). Then antagonist activity of tamoxifen toward β-estradiol was examined. It has been reported that the affinity of tamoxifen to estrogen receptor is much lower than that of β-estradiol (19). Thus, although tamoxifen inhibited growth of MCF-7 cells below control level at concentrations higher than 10^{-8} M, the inhibitory effect could be prevented by simultaneous addition of as little as 1000-fold less β-estradiol. Taking into account these results, we used β-estradiol at a concentration of 10^{-12} M, at which both [3H]thymidine incorporation and the amount of pS2 protein in the medium were increased about 3-fold (data not shown). As shown in Fig. 2, simultaneous addition of tamoxifen inhibited stimulation of DNA synthesis and induction of pS2 protein achieved by β-estradiol alone in a dose-dependent manner, indicating the involvement of estrogen receptor in these effects. Another antiestrogen, nafoxidine, gave essentially the same results (data not shown).

As shown in Fig. 3, simultaneous addition of cycloheximide (10 μg/ml) or actinomycin D (10 ng/ml) with β-estradiol (10^{-8} M) completely abolished both stimulation of DNA synthesis and induction of pS2 protein by β-estradiol alone, indicating that both effects require new RNA and protein synthesis.

Time Course of the Effects of β-Estradiol. To obtain information about the relationship between cell growth and production of pS2 protein, MCF-7 cells treated for 24 h with MEM-BSA were cultured in the presence or absence of β-estradiol (10^{-8} M) for various times (Fig. 4). [3H]Thymidine incorporation into DNA in the absence of β-estradiol slightly increased for several hours probably due to the effect of the medium change. Stimulation of [3H]thymidine incorporation was clearly observed from 6 h after the start of treatment of the cells with β-estradiol as compared with control cells. The leveling off in [3H]thymidine incorporation in the cells cultured with β-estradiol for several hours from 21 h is most likely due to a round of cell cycle. pS2 protein in the medium began to accumulate from 12 h after the start of treatment of the cells with β-estradiol as compared with control cells. Thus the increase in DNA synthesis preceded the increase in the amount of pS2 protein in the medium.

Mitogenic Activity of pS2 Protein. To test whether pS2 protein stimulates cell growth directly, purified pS2 protein was added to MCF-7 cells that had been kept in MEM-BSA for 24 h. As shown in Fig. 5A, however, after a 24-h incubation [3H]thymidine incorporation into DNA was not increased significantly by pS2 protein, whereas human EGF stimulated [3H]thymidine incorporation in a dose-dependent manner at the same concentration as pS2 protein. The amount of pS2 protein protein was increased about 3-fold at concentrations of 10^{-8} M. This suggests that neither DNA synthesis nor cell growth was induced by pS2 protein alone. Therefore, the effects of pS2 protein on cell growth and DNA synthesis require the participation of new RNA and protein synthesis. As shown in Fig. 5B, human EGF stimulated [3H]thymidine incorporation 30-fold at the concentration of 1 ng/ml, pS2 protein did not stimulate that at all up to 100 ng/ml.
Corticoid, insulin, and EGF (17, 18, 22-24). The cells respond with high affinity for estrogen, androgen, progesterone, glucocorticoid also did not induce pS2 protein in spite of the presence of their receptors in the cells (22, 23).

Antiestrogens such as tamoxifen compete with estrogen for binding to the estrogen receptor (19). In mammals, tamoxifen acts estrogically as well as antiestrogenically depending on the response studied. In MCF-7 cells, it has been reported that tamoxifen is able to induce progesterone receptor as β-estradiol does (30), whereas it antagonizes induction by β-estradiol of specific proteins (M, 52,000 and 160,000) and pS2 mRNA (31). Also in our system, although tamoxifen alone did not cause significant alteration of the amount of pS2 protein in the medium, it inhibited induction of pS2 protein by β-estradiol in a dose-dependent manner (Fig. 2). Induction of pS2 protein by β-estradiol required new mRNA and protein synthesis (Fig. 3).

DISCUSSION

MCF-7 cells were initially derived from malignant effusions of a woman with metastatic breast cancer (20) and extensively characterized with respect to their human and breast origin (21). MCF-7 cells are well known to possess specific receptors with high affinity for estrogen, androgen, progesterone, glucocorticoid, insulin, and EGF (17, 18, 22-24). The cells respond to estrogen, androgen, insulin, and EGF with an increased rate of DNA, RNA, and protein synthesis (17, 18, 23, 24). Among these, stimulation of cell growth and induction of various proteins by estrogen are well documented (Ref. 25 and references therein).

Jakowlew et al. (12) isolated and characterized a cDNA clone of about 600 nucleotides corresponding to an mRNA (pS2) which accumulated in response to estrogen treatment of MCF-7 cells. Activation of transcription of the pS2 gene is a primary response to estrogen (26), and the gene encodes an 84-amino acid polypeptide (12). Secretion of the pS2 gene product into the medium in response to estrogen was demonstrated by immunoprecipitation (27) and our previous results revealed that the product is secreted as a mature polypeptide composed of 60 amino acids after the signal polypeptide cleavage (11). Thus pS2 protein is a cysteine-rich low molecular weight polypeptide produced and secreted by cancer cells. These characteristic features suggest that possibly it is an estrogen-inducible growth factor and confers autonomous growth on cancer cells. Davidson et al. (28) examined the expression of the pS2 gene in two variants of MCF-7 cells. In the variant cell line 113, the cell growth was inhibited but the pS2 mRNA was still induced by estrogen treatment. In the variant cell line LY2, while the cells were resistant to the growth-inhibitory effect of an antiestrogen (LY117018) and grew normally even in the presence of LY117018, the expression of the pS2 gene was inhibited by LY117018. These results suggest that pS2 protein is not a major autocrine growth-stimulating agent.

In the present study, we investigated extensively the relationship between production of pS2 protein and growth of MCF-7 cells when the cells were treated with various hormones. We demonstrated that pS2 protein is induced specifically by physiological concentrations of β-estradiol which stimulate cell growth (Fig. 1A). The weak induction of pS2 protein by testosterone (Fig. 1B) may result from the fact that androgen interacts with estrogen receptor only at high concentrations (29). In contrast, progesterone stimulated neither cell growth nor synthesis/secretion of pS2 protein (Fig. 1C). This results matches the previous finding (22) that progesterone receptors present in MCF-7 cells bind progesterone but cannot transduce the information probably due to the lack of ability to translocate to the nucleus. Insulin and glucocorticoid also did not induce pS2 protein in spite of the presence of their receptors in the cells (22, 23).
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Fig. 4. Time course of effects of β-estradiol. After a 24-h incubation in MEM-BSA, the cells were cultured in fresh MEM-BSA in the absence or presence of β-estradiol (10⁻⁸ M) for the various times indicated on the abscissa. Acid-insoluble radioactivity incorporated into the cells (A) and the amount of pS2 protein in the medium (B) were determined as described in “Materials and Methods.” Values are expressed as the means ± SE (bars) of three determinations.

Fig. 5. Mitogenic activity of pS2 protein. Purified pS2 protein or human EGF at the concentration indicated on the abscissa was added to the culture medium of MCF-7 cells that had been kept in MEM-BSA for 24 h (A) or BALB/c 3T3 cells that had been kept in Dulbecco’s modified MEM containing 0.5% FCS for 48 h (B). After a 23-h incubation, 1 µCi/well of [³H]thymidine was added to the medium and the cultures were further incubated for 1 h. Acid-insoluble radioactivity incorporated into the cells was determined as described in “Materials and Methods.” Values are expressed as the means ± SE (bars) of three determinations.

Thus, after binding to its specific receptor, estrogen activates transcription of the pS2 gene, which culminates in the accumulation of pS2 protein in the medium.

These data based on the amount of pS2 protein in the culture medium are in good agreement with the previous results on expression of the pS2 gene at the mRNA level. Since our enzyme immunoassay system delineates satisfactorily the behavior of pS2 protein in cells receiving various hormones, this system is verified to detect only pS2 protein in the case of MCF-7 cells.

The fact that stimulation of cell growth and induction of pS2 protein were obtained by the same concentrations of β-estradiol raised the possibility that pS2 protein is an autocrine growth factor responsible for the growth-stimulating effect of β-estradiol. However, differential time treatment of the cells with β-estradiol revealed that the increase in the amount of pS2 protein in the medium followed the increase in DNA synthesis (Fig. 4). Stimulation of [³H]thymidine incorporation was clearly observed from 6 h and accumulation of pS2 protein in the medium began from 12 h after the start of treatment of the cells with β-estradiol as compared with control cells. These results seem to deny the mechanism that pS2 protein induced by β-estradiol is then secreted into the medium to cause the stimulation of DNA synthesis after binding to its cell surface receptor. Increase in [³H]thymidine incorporation from 6 h observed in Fig. 4 appears to be early as compared with previous reports, e.g., by Bronzert et al. (32), where the increase was observed after 12 h estrogen treatment. The difference in the time course indicates that culture of the cells in MEM-BSA for 24 h did not bring them to complete quiescence and may be ascribed to the presence of phenol red which acts as a weak estrogen (15).

Furthermore, direct addition of purified pS2 protein did not alter [³H]thymidine incorporation into either MCF-7 or BALB/c 3T3 cells significantly, although human EGF stimulated DNA synthesis as reported (24) (Fig. 5). Thus far in our examinations, we did not obtain any results showing mitogenic activity of pS2.

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protein for cultured cells including human gastric cancer cells (MKN-1, MKN-28, MKN-45, and MKN-74). From these results together with the following findings, we suppose that pS2 protein has no mitogenic activity. (a) Mitogenic activity toward MCF-7 cells or BALB/c 3T3 cells detected in serum-free conditioned medium of MCF-7 cells was not recovered in the elution position of pS2 protein after gel filtration on a Sephadex G-50 column, indicating that mitogenic activity in the medium is due to growth factors other than pS2 protein, which are known to be secreted by MCF-7 cells (33–35). (b) Final purification of pS2 protein was achieved by reverse-phase high-performance liquid chromatography using acetonitrile and trifluoroacetic acid (11). In general, mitogenic activities of polypeptide growth factors such as EGF and platelet-derived growth factor are stable even after reverse-phase high performance liquid chromatography probably due to the presence of multiple disulfide bonds. This is maybe the case of pS2 protein which contains three disulfide bonds (11). (c) DNA synthesis of MCF-7 cells stimulated by β-estradiol, EGF, or insulin at various doses was not altered by coincubation of purified pS2 protein, indicating no toxicity of the remaining trace of acetonitrile and trifluoroacetic acid in purified pS2 protein. The results also suggest that cooperation of pS2 protein and other estrogen-inducible factors did not work well for growth stimulation.

Despite the findings mentioned above, however, we cannot rule out the possibility that the biological activity of pS2 protein was lost during the purification procedure. It is also possible that the lack of mitogenic activity in purified pS2 protein is due to the loss of cofactor(s) such as another hormone or binding protein required for the activity of pS2 protein.

Our findings in this report confirm the results reported by Davidson et al. (28) with another methodology and suggest that induction of pS2 protein by estrogen is not involved in the growth-stimulating effect of estrogen in MCF-7 cells. The biological significance of pS2 protein still remains unknown.

REFERENCES

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