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HORMONAL REGULATION OF c-*erb*B-2 ONCOGENE EXPRESSION IN BREAST CANCER CELLS

M. DE BORTOLI,* C. DATI, S. ANTONIOTTI, P. MAGGIORA and M. L. SAPEI
Laboratory of Molecular Cell Biology, Department of Animal Biology, University of Turin, Via Acc. Albertina 17, 10123 Torino, Italy

Summary—Expression of the c-*erb*B-2 (*neu*, HER-2) oncogene is found to be subjected to hormonal and developmental regulation in normal as well as neoplastic mammary cells. We have previously reported that estrogens inhibit c-*erb*B-2 expression at both the mRNA and protein level in estrogen receptor (ER)-positive, but not in ER-negative, breast cancer cell lines. Reversion of c-*erb*B-2 inhibition is seen with tamoxifen. The effect on c-*erb*B-2 expression of several other hormones and factors, which influence mammary cell growth and differentiation, has been studied. Our observations indicate that, in normal and neoplastic mammary cells, c-*erb*B-2 expression is inversely related to cell proliferation. While estrogens, anti-estrogens and cAMP clearly regulate c-*erb*B-2 mRNA levels, epidermal growth factor dramatically decreases the c-*erb*B-2 protein without affecting the level of c-*erb*B-2 mRNA. Therefore, different signals converging in terms of cell proliferation regulate c-*erb*B-2 expression by different molecular mechanisms.

INTRODUCTION

Among all known oncogenes, c-*erb*B-2 (also indicated as HER-2 and *neu*) is probably the most important oncogene in breast cancer. The c-*erb*B-2 gene is in fact amplified and/or overexpressed in 20–25% of primary human breast carcinomas and, what is more relevant, its alteration is associated with a particularly unfavorable clinical outcome [1–3]. c-*erb*B-2 amplification and expression, which are assayable in tumor biopsies, are now under evaluation as new prognostic factors for breast cancer [4].

c-*erb*B-2 encodes a 185 kD protein (p185), which is a member of the family of transmembrane growth factor receptors with tyrosine-specific protein kinase activity. c-*erb*B-2 presents a very high homology to, but is distinct from, the epidermal growth factor receptor (EGFR) [5–7]. A 35 kD glycoprotein has been reported as the ligand for p185 [8, 9], but the complete characterization of its biological and biochemical activity has not been accomplished yet.

Experimentally, it has been proved that structural alterations to the c-*erb*B-2 gene, leading to increased and unregulated activity of the c-*erb*B-2 tyrosine kinase, activate its oncogenic potential. These alterations are represented by point-mutations in the trans-membrane domain [10,11], or by gene amplification, leading to constitutive overexpression. In experimental model systems, the latter condition can be represented by the integration of c-*erb*B-2 cDNA driven by constitutive promoters [12]. The activated c-*erb*B-2 has been shown to transform NIH3T3 fibroblasts [12, 13], to induce mammary neoplasia in transgenic mice [14, 15] and to increase the oncogenic potential of human breast cells [16].

In contrast to the wide information available on the oncogenic activity of c-*erb*B-2, very little is known about its functional role and about the mechanisms controlling its expression in mammary cells. Discovering the functions and the regulation of c-*erb*B-2 in breast cancer cells is particularly important since, from this knowledge, novel therapeutical tools could be designed to either impair functioning of the c-*erb*B-2 protein, or suppress its expression in tumor cells.

The promoter region of human c-*erb*B-2 has been identified [17] and transcription from this promoter shown to be increased by EGF, cAMP, phorbolesters and retinoic acid in HeLa cells [18].

In the normal mammary gland, the level of the c-*erb*B-2 protein increases during the last steps of the functional development in pregnant rats [19] and the presence of an

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*To whom correspondence should be addressed.

activated c-*erb*B-2 oncogene does not interfere with hormonal induction of β-casein in the normal mammary epithelial cell line HC-11 [20]. These results suggest a role of c-*erb*B-2 in the functional differentiation of mammary cells.

We and others have previously reported that the expression of c-*erb*B-2 is inhibited by estrogens in human breast cancer cells [19, 21]. This estrogenic effect is limited to estrogen receptor-positive (ER+) cells, is appreciable within hours from estrogen treatment and parallels stimulation of cell proliferation.

In this article, we summarize the results obtained in our laboratory, further characterizing the regulation of c-*erb*B-2 expression in human breast cancer cells by hormones and factors that affect mammary cell growth and differentiation. Our data point out that the expression of c-*erb*B-2 is controlled by multiple factors and that its regulation is achieved through different molecular mechanisms.

Estrogens inhibit c-erbB-2 expression

We have previously reported that estrogen treatment of ER+ human breast cancer cells leads to a strong decrease of both c-*erb*B-2 mRNA and protein levels [19]. The inhibitory effect of estrogens was observed on MCF7 cells and on the estrogen-responsive variant of the T47D cell line. To further define the specificity of this effect, we have studied the effect of 17β-estradiol and of the anti-estrogenic compounds tamoxifen and 4-hydroxy-tamoxifen on c-*erb*B-2 expression, using one additional ER+ cell line, ZR75.1, and two ER− lines, SKBR3 and MDA.MB.231. The use of an additional ER+ cell line was necessary since both T47D and MCF7 cells, which were used in the previous study, present anomalies affecting the c-*erb*B-2 oncogene. In fact, our T47D cell present a slightly amplified c-*erb*B-2 gene copy number, as determined by Southern blot analysis, and MCF7 cells have been reported to express an anomalous c-*erb*B-2 transcript, most likely due to an additional RNA start site present in the c-*erb*B-2 gene of these cells [17].

On the other hand, SKBR3 and MDA.MB.231 cells were chosen to represent ER− lines since they present a very high and very low c-*erb*B-2 expression, respectively. In SKBR3 cells, c-*erb*B-2 overexpression is due to the presence of several copies of the gene [22].

The data obtained in our laboratory by Northern blot and immunoblotting analysis consistently demonstrated that 17β-estradiol depresses both c-*erb*B-2 mRNA and protein levels, in all the ER+ cell lines tested, whereas it has no effect on the ER− cell lines SKBR3 and MDA.MB.231. In all ER+ cells, but not in ER− cells, the anti-estrogens tamoxifen and 4-hydroxy-tamoxifen were able not only to prevent c-*erb*B-2 inhibition by 17β-estradiol, but also to rapidly induce c-*erb*B-2 mRNA and protein expression in cells cultured in medium containing untreated fetal calf serum. Figure 1 gives an example of estrogen and tamoxifen effects on c-*erb*B-2 mRNA and protein expression, relative to the T47D cell line.

In all cases, inhibition of c-*erb*B-2 expression by estrogens in ER+ cells was accompanied by stimulation of cell growth (see Table 1). No effects of either 17β-estradiol or tamoxifen and 4-hydroxy-tamoxifen were seen on the growth of ER− cell lines.

Our results demonstrate that inhibition of c-*erb*B-2 expression by estrogens in human breast cancer cells depends on the presence of ERs. Time-course studies on ZR75.1 cells have shown that the reduction of c-*erb*B-2 mRNA level is appreciable within 4–6 h after estrogen treatment. In addition, recent results obtained in our laboratory using cycloheximide have shown that c-*erb*B-2 inhibition by estrogens requires protein synthesis, suggesting that the action of estrogens on c-*erb*B-2 is indirect.

c-erbB-2 regulation by other hormones

The possible regulation of c-*erb*B-2 expression by other hormones and factors that are known to affect mammary cell growth and differentiation was studied, using T47D, ZR75.1 and SKBR3 cells. In addition, regulation of c-*erb*B-2 was also studied using the HC-11 cell line, which are normal mouse mammary epithelial cells, capable of differentiation "*in vitro*" [23].

The effect of progestins, corticosteroids, insulin and prolactin, hormones that cooperatively and sequentially regulate the differentiation of the mammary gland during pregnancy, was examined. Effects on c-*erb*B-2 mRNA and protein expression were measured in the presence or absence of an estrogenic milieu. As shown in Table 1, none of these hormones demonstrated dramatic effects on either cell growth or c-*erb*B-2 expression. A slight growth inhibitory action of progestins and prolactin was accompanied by slightly increased c-*erb*B-2 expression. Wider effects on c-*erb*B-2

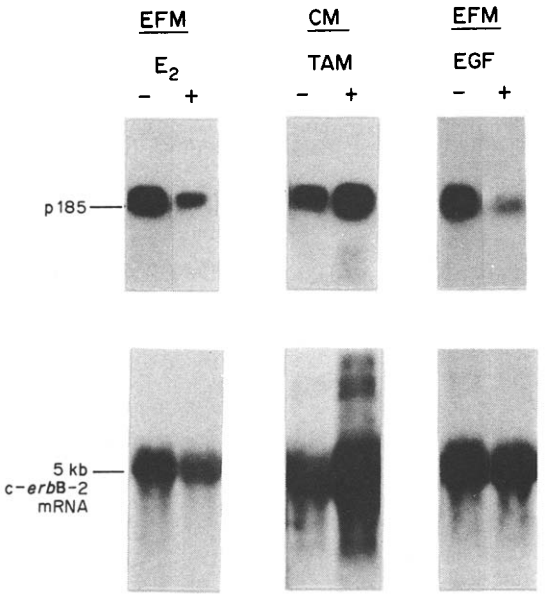

Fig. 1. Effects of 17β-estradiol, 4-hydroxy-tamoxifen and EGF on p185 (upper panel) and c-*erb*B-2 mRNA levels (lower panel) in T47D cells. CM, cells kept in 95% DMEM and 5% FCS (complete medium); EFM, cells plated 24 h in complete medium and then switched for 3 days in phenol red-free EMEM containing 5% charcoal-stripped FCS (estrogen-free medium). p185 levels were analyzed on 100 μg aliquots of cell lysates, resolved on 8% polyacrylamide–SDS gels and immunoblotted with anti-21N anti-serum, as previously described [19]. Total RNA were extracted by the guanidine/LiCl method, and electrophoresed through 1.2% agarose-formaldehyde gels. Northern blots were hybridized with a 1.1 kb BamHI 5′ fragment from the pCER204 human cDNA [7].

expression were seen in the case of EGF and cAMP.

EGF's mitogenic action on both normal and transformed mammary cells is well characterized. EGF is also an essential competence factor for mammary differentiation "*in vitro*" [20]. Conversely, derivatives of cAMP, such as dibutyryl-cyclic-AMP (DBcAMP), have been shown to exert an anti-proliferative effect on human breast cancer cells [24]. As shown in Table 1, DBcAMP increased both c-*erb*B-2 mRNA and protein levels, at concentrations inhibiting cell growth. EGF showed mitogenic activity on all cell lines tested, accompanied by a strong reduction of c-*erb*B-2 protein level. Noteworthy, EGF did not reduce c-*erb*B-2 mRNA levels, the implications of which will be discussed later.

c-*erb*B-2 expression was observed to be also regulated in a density-dependent fashion: in both T47D and HC-11 cells, c-*erb*B-2 expression was enhanced in cells reaching confluence and hence attaining a growth resting phase. EGF-primed, confluent HC-11 cells can be induced to produce β-casein by the combined action of dexamethasone, insulin and prolactin [23]. During this differentiation phase in HC-11 cells, the c-*erb*B-2 protein, but not mRNA, is further increased (our results and N. E. Hynes, personal communication), indirectly confirming the previous finding of high c-*erb*B-2 expression in the lactating rat mammary gland [19].

A summary of results, shown in Table 1, suggests that the expression of the c-*erb*B-2 oncogene is inversely related to the proliferative activity in mammary cells. Expression of the c-*erb*B-2 protein is in fact enhanced during growth arrest and/or differentiation and depressed in rapidly proliferating cells.

c-erbB-2 is regulated by different molecular mechanisms

The data summarized here suggest that the regulation of c-*erb*B-2 expression in mammary cells is achieved by two different molecular mechanisms. c-*erb*B-2 regulation by estrogens and DBcAMP, as well as the slight effects observed with progestins and prolactin, depends on mRNA regulation at either transcriptional or post-transcriptional level. Transcriptional

Table 1. Effects of different treatments on c-*erb*B-2 mRNA and p185 levels and cell proliferation on different mammary cell lines

Cell line	Treatment	c-*erb*B-2 mRNA	p185	Cell growth
T47D, MCF7, ZR75.1	Estradiol	Decreased	Decreased	Enhanced
	Tamoxifen	Increased	Increased	Inhibited
T47D, ZR75.1, HC11, SKBR3	EGF	Unchanged	Decreased	Enhanced
T47D, HC11, ZR75 1	Progesterone	Unchanged	Small increase	Small inhibition
	Prolactin	Unchanged	Small increase	Small inhibition
	Insulin	Unchanged	Unchanged	Small increase
	Cortisol	Unchanged	Unchanged	Unchanged
ZR75.1	DBcAMP	Increased	Increased	Inhibited

c-*erb*B-2 mRNA was analyzed by Northern blotting and p185 levels by immunoblotting as described in Fig 1. Cell growth was evaluated by Burton's method of DNA assay. Treatments were at the following concentrations. estradiol: 10 nM, 4-hydroxy-tamoxifen: 1 μm, EGF (human): 10 ng/ml, progesterone (R5020): 10 nM, prolactin (human): 100 ng/ml, insulin: 10 μg/ml, cortisol: 10 nM, and DBcAMP 1 mM.

regulation seems more likely, since effects of cAMP and other differentiating agents on the c-*erb*B-2 promoter have been described [18]. Studies on estrogen and cAMP influence on c-*erb*B-2 transcription rate are under way in our laboratory.

The mechanism of action presented by EGF is notably different. EGF treatment of both ER+ and ER− cell lines resulted in a rapid drop of the c-*erb*B-2 protein. However, Northern blot analysis clearly showed that c-*erb*B-2 mRNA levels are not affected by EGF treatment. EGF stimulation of the c-*erb*B-2 promoter has been reported [18]. It is possible that the stable c-*erb*B-2 mRNA level seen in mammary cells under EGF treatment is the result of two opposite effects: inhibition, as a generic effect of growth enhancement; stimulation, as a specific EGF effect on the c-*erb*B-2 promoter [18].

EGF effect on the c-*erb*B-2 protein, p185, is probably mediated through direct interaction of the two similar receptor proteins. EGF-dependent tyrosine phosphorylation of p185 has been reported [25] and evidence that p185 and the EGF receptor can physically interact to produce heterodimeric forms of altered stability have been presented [26]. Thus, one possibility is that EGF decreases p185 half-life through p185/EGF dimerization followed by rapid internalization and degradation. This possibility is currently being investigated in our laboratory.

CONCLUSIONS

The above summarized observations indicate that, in normal and neoplastic mammary cells, the expression of the c-*erb*B-2 oncogene is inversely related to cell proliferation. Several signals related to cell proliferation and/or differentiation appear to converge to regulate c-*erb*B-2 expression at the mRNA level. In sharp contrast, EGF down-regulates p185 without affecting mRNA levels, suggesting that EGF activates a different metabolic pathway, allowing stable c-*erb*B-2 mRNA expression, even in rapidly proliferating cells.

Since c-*erb*B-2 amplification is present in the most aggressive breast tumors, it may be surprising that c-*erb*B-2 expression is inversely related to cell proliferation. However, based on our findings, it may be hypothesized that down-regulation of p185 represents an essential step of mitogenic stimulation, in normal mammary cells. The amplified c-*erb*B-2 gene copy number frequently found in breast cancer cells most likely drives a constitutively elevated c-*erb*B-2 expression. Inability to repress c-*erb*B-2 expression during mitogenic stimulation may represent one anomalous metabolic condition contributing to the malignant phenotype.

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