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The Unliganded Estrogen Receptor (ER) Transduces Growth Factor Signals

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In the absence of serum and estrogen, we show that the growth of the prolactin secreting pituitary tumour cell line, GH_3 is stimulated by insulin and insulin-like growth factor-1 (IGF-1) and this response is blocked by the steroidal antiestrogens, ICI 164384 and ICI 182780. From conditioned medium (CM) experiments, growth of low density cells (10k/cm^2) is increased by the addition of CM from high density cells (100k/cm^2) and this growth effect is also blocked by antiestrogen. Transfection studies with a ΔMTV-ERE-LUC reporter plasmid show that in the absence of estrogen and serum, both insulin and IGF-1 induce luciferase expression and this is blocked by the pure antiestrogens. No effect of these treatments was apparent when parallel experiments were conducted with a plasmid construct lacking the vitellogenin estrogen response element. From these and other data discussed in this report, we conclude that for GH_3 cells, in the absence of estrogen and serum, the ER is transcriptionally activated by intracellular peptide factor pathways and by this means, acts as the key nuclear factor inducing mitogenesis in response to autocrine and exogenously added growth factors.

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INTRODUCTION

The first event associated with mitosis induced by growth factors is the activation of the cell surface receptor by tyrosine phosphorylation. Phosphorylated tyrosine residues of the intracellular portion of the receptor are then used as docking sites for a number of cytosolic proteins with regions (SH2 domains) homologous to the c-*src* protooncogene product [1]. These proteins continue the signalling cascade, generating diverse signals depending on the cell type and environment. How these signals are transmitted to the nucleus is poorly understood, but the first nuclear event associated with growth factor action is the induction of the protooncogene c-*fos* [2]. This gene is also induced by a number of other substances that give rise to a mitogenic response. Included amongst these is estradiol (E_2) [3]. For a growth response to E_2, it would appear that growth factors, like insulin and insulin-like growth factor-1 (IGF-1) are almost an absolute requirement in some cell systems [4]. Conversely it has been shown that E_2 increases the growth stimulatory effect of IGF-1 on the breast cell line MCF-7 [5]. It would appear from these data that the estrogen receptor (ER) is essential for the maximum mitogenic effect of IGF-1. The interdependency of these two signalling pathways suggests a common pathway of growth induction. Recently evidence has been accumulating for a cross-talk between peptide and steroid pathways. Therefore based on the observation that the signalling pathway for the dopamine D1 receptor involves the progesterone receptor [6, 7], this might be achieved for the ER by a direct transcriptional activation of the receptor by the IGF-1 response pathway. Alternatively the ER could interact with other transcription factors induced by IGF-1 in an analogous manner to Fos–Jun proteins that form the AP-1 complex [8]. Here we describe studies designed to test the idea that the ER is one of the nuclear factors responsible for the mitogenic effects of growth inducing peptides.

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EXPERIMENTAL

Chemicals

Materials and reagents, except where stated, were obtained from Gibco (Karlsruhe, Germany), Flow (Meckenheim, Germany), Falcon (Heidelberg, Germany), Nunc (Weisbaden, Germany) and Sigma (Deisenhofen, Germany). E_2 was dissolved in ethanol

to give a stock solution of 1 mg/ml. The pure steroidal antiestrogens, ICI 182780 and ICI 164384 were obtained from Zeneca Pharmaceuticals (Cheshire, England) and their purity was checked before use by HPLC using a C18 reverse phase column and methanol–water (80:20) as eluent. Stock solutions of both compounds, prepared in ethanol at a concentration of 20 mM, eluted essentially as one peak, with retention times of 9.1 min for ICI 164384 and 5.3 min for ICI 182780. Human insulin was obtained from Novo Industries (Mainz, Germany) and human IGF-1 was obtained from Peninsula Labs (Heidelberg, Germany).

Cell culture

The ER containing pituitary tumour cell line GH_3 [9] was obtained at passage 21 from the American Type Culture Collection (Rockville, MD, U.S.A.) and maintained as a stock culture in Dulbecco's modified essential medium (DMEM) with 10% foetal calf serum (FCS). Prior to experiments, these cells were adapted for at least 2 weeks (4 passes) in phenol red free IMEM containing transferrin (30 μg/ml), bovine serum albumin (BSA) (1 mg/ml) cholesterol (14 μg/ml), soy-bean lipids (60 μg/ml) (Vitromex, Vilshofen, Germany) and 0.5% charcoal stripped FCS (SFCS). The latter was prepared by the addition of activated charcoal (Sigma, C-4386) to FCS (final concentration 0.1% w/v) and mixing over a period of 1 h at 37°C. The charcoal was then sedimented by centrifugation and the supernatant was sterilized by passing through a 0.2 μm filter unit. For growth experiments, adapted cells were seeded into multiwell plates (10 k/cm^2) in the above medium and left for 72 h. Following this period, the medium was twice changed over a 48 h period with serum free IMEM and then cells were treated with insulin, IGF-1, antiestrogens and E_2. Cell numbers were determined directly by the use of a Coulter counter or by the addition of the tetrazolium dye, MTT, as described previously [10]. In brief following the treatment period, MTT was added to serum free medium to give a final concentration of 0.01 mg/ml. Cells were then left at 37°C for 4 h and the blue crystals formed were solubilized overnight at 37°C by the addition of SDS in HCl to give final concentrations of 10% and 0.01 M, respectively. Colour development was then quantitated at 550 nm using a multiwell scanner (Dynatech) after transfer of 300 μl aliquots from each treatment well to 96 well plates. Prior estimations under a number of different growth condition indicated that 0.100 absorbance units corresponded to around 50,000 cells. Antiestrogen addition to cell cultures was made such that the final concentration of ethanol was 0.1%, an amount that had no discernable effect on growth.

Prolactin measurements

Prolactin concentrations were measured in GH_3 cell conditioned medium using a radioimmunoassay (RIA) as described previously [11]. In brief cells were seeded into 24 well plates at a density of 40 k/cm^2 in IMEM containing 0.5% SFCS. After 72 h this medium was changed twice over 48 h for serum free IMEM and then cells were treated in the same medium for 72 h. Following this the medium was removed and replaced with fresh treatment medium. After a further 24 h this medium was removed and stored at -20°C for prolactin RIA and the cell number for each well was estimated using the MTT assay.

Transfection studies

Transfections of pituitary GH_3 cells were carried out using an electroporation system (Biotechnologies and Experimental Research Inc. San Diego, CA). After determination of the optimal electric field strength [12], 5 μg of the reporter plasmids ΔMTV-ERE-LUC and ΔMTV-LUC (the construction of these is described below) and 5 μg carrier DNA (pGEM4; Promega Corp., Madison, WI) were cotransfected with 5 μg of pCH110 — a plasmid containing the β-galactosidase gene downstream of a SV40 early promotor (Pharmacia, LKB, Freiburg, Germany). Electroporated cells were replated in phenol red and serum free IMEM and incubated immediately with various treatments. After 24 h, cells were harvested and extracts were assayed for luciferase activity [13] and β-galactosidase activity [14] to control transfection efficiency.

The ΔMTV-ERE-LUC construct [15] used here was made by inserting a palindromic vitellogenin estrogen response element (ERE) (5′ TCAGG-TCACAGTGACCTGA-3′) [16] into the unique HindIII site of the ΔMTV-CAT plasmid in which major GREs located between positions -190 and -88 of the MTV-LTR were deleted, thus generating ΔMTV-ERE-CAT. Subsequently, the CAT coding sequence was replaced with the luciferase coding sequence obtained from pSVOA/L-AΔ5′ [13] giving ΔMTV-ERE-LUC.

RESULTS

Under serum and estrogen deprived conditions GH_3 cells grew with doubling times longer than under stock culture conditions (2.3 ± 0.05 days vs 1.7 ± 0.1 days; mean $\pm$ SD for three different passes). For a number of experiments where cells were maintained in the absence of serum and estrogen, insulin and IGF-1 increased cell growth in a concentration dependent manner. A typical experiment is shown in Fig. 1(a). Figure 1(b) shows the block of basal and IGF-1 induced growth by 10^{-8} M ICI 182780 and its reversal by a range of E_2 concentrations. It should be noted that for all experiments conducted under serum free conditions during this study, E_2 over a concentration range from 10^{-13} to 10^{-7} M failed to stimulate the growth of these cells in the absence or presence of IGF-1 or insulin. In the absence of exogenous growth factors, it is clear from Fig. 1(b) that the antiestrogen inhibited the basal growth rate to between 50–60% of the untreated control values. This block and that of the growth induced by IGF-1 is clearly reversed by E_2, reflecting the affinity of this steroid for the ER [17]. At the maximum

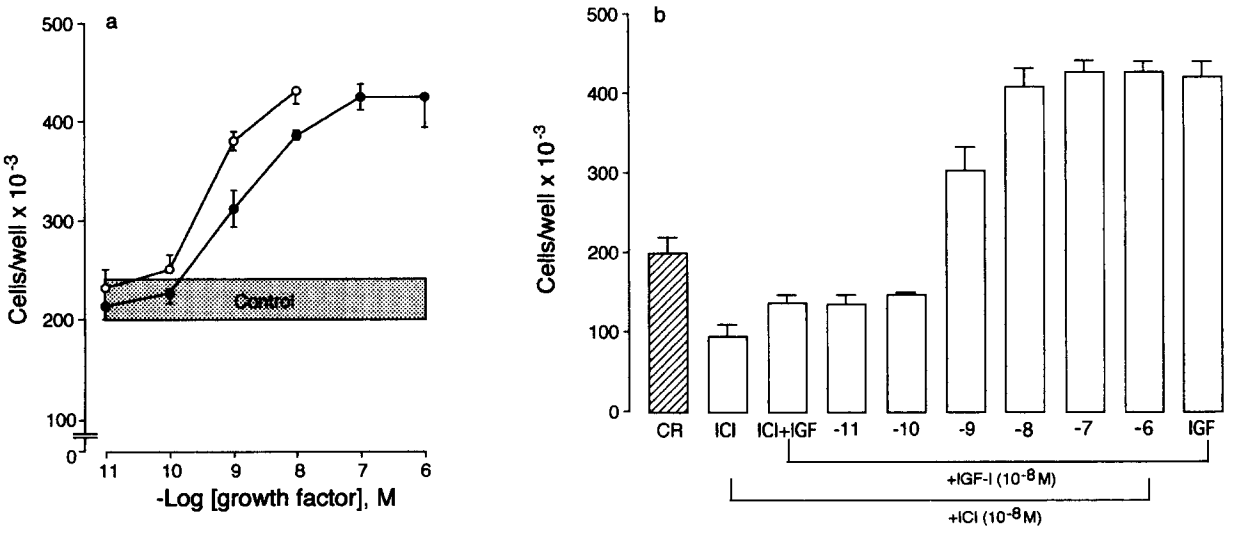

Fig. 1. Serum and estrogen free growth stimulation of GH_3 cells by insulin and IGF-1 and block of this effect by ICI 182780. (a) Following seeding and medium changes as described in Experimental, cells were treated with a concentration range of IGF-1 (—○—) or insulin (—●—) and allowed to grow for 5 days before harvesting. All values for cell number are shown as mean ± SD. At the time of treatment, cell numbers estimated in parallel wells, were 46,000 ± 7000. (b) ICI 182780 was added to cells alone or in combination with IGF-1 (10 nM) and a concentration range (10^{-11} to 10^{-6} M) of E_2.

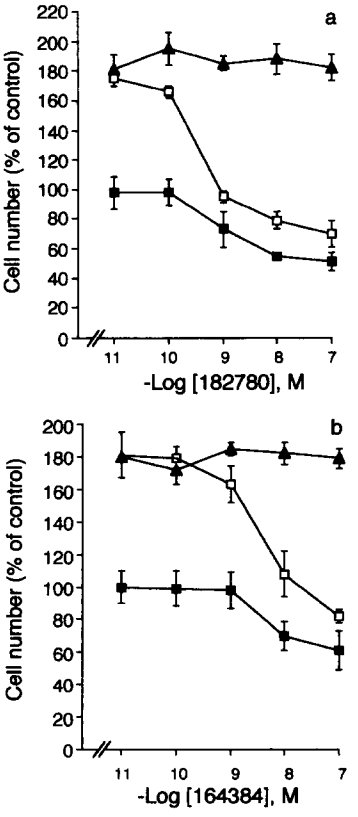

Fig. 2. Dose inhibitory effects of the steroidal antiestrogens ICI 182780 (a) and ICI 164384 (b) in the presence of maximal growth stimulatory concentration of IGF-1 (10 nM). Cells were treated with a range of anti-estrogen concentrations alone (—■—), with antiestrogen and 10 nM IGF-1 (—□—) or with antiestrogen, IGF-1 and 100 nM E_2 (▲). Values are expressed as percentage of the no treatment control. The error bars represent the CV for the untransformed data at each treatment point. IC_{50} values for ICI 182780 and ICI 164384 in the presence of IGF-1 were determined as 0.6 nM and 6 nM, respectively.

growth stimulatory concentration of IGF-1 (10 nM), Fig. 2 shows the dose responses to ICI 182780 and ICI 164384. Both compounds effectively blocked the growth effect of IGF-1 with IC_{50} values similar to those reported for their antiestrogen effects in other systems [18].

The clear ability of the antiestrogens to interfere with the basal growth rate of these cells in the absence of estrogen and serum was tested using medium conditioned for 24 h by high density GH_3 cells (100 k/cm^2). As Fig. 3 shows, the basal growth rate of GH_3 cells,

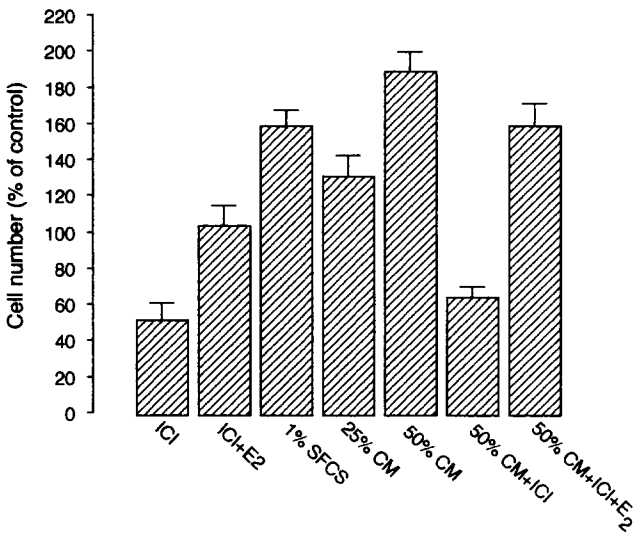

Fig. 3. The effect of serum and estrogen free CM, produced by high density GH_3 (100 k/cm^2) cells, on the growth of GH_3 cells seeded at low density (10 k/cm^2). Cells were seeded into 48 well plates at a density of 10 k/well and treated as indicated following the serum free washout. Cell numbers were indirectly estimated by the use of the MTT method. SFCS = charcoal stripped FCS.

when seeded at low density is markedly increased by the addition of conditioned medium (CM) from the high density cells and this is blocked by antiestrogen.

It might be argued that the effects of antiestrogen on IGF-1 and insulin induced growth, in the absence of an effect of E_2, represents a non-specific interference of the antiestrogen-liganded ER with transcription of essential growth promoting genes. Since the GH_3 cell line secretes prolactin [19] and the gene for this hormone is under the control of the ER [20], we have attempted to address the above by determining the effect of ICI 182780 and E_2 on prolactin levels in conditioned medium under a protocol that reflects its rate of synthesis. Table 1 shows that in contrast to growth, E_2 markedly increases the concentration of prolactin detected in CM and this increase and that of the basal secretion, is reduced to below the detection limit of the RIA (0.5 ng/ml).

To determine whether the effects on growth of insulin and IGF-1 in combination with antiestrogen reflected changes in the transcriptional state of the estrogen free ER, cells were transfected under serum and estrogen free conditions with reporter plasmids containing or lacking the vitellogenin ERE. Over five experiments where cells were transfected with the ΔMTV-ERE-LUC reporter, treatment with E_2 or insulin and IGF-1 resulted in a marked induction of luciferase activity (1.6- to 4-fold, E_2; 1.4- to 7-fold, insulin and IGF-1) above untreated controls and this was completely blocked by cotreatment with antiestrogen. Results for one typical experiment where IGF-1 was used are shown in Fig. 4. In contrast, no induction of luciferase expression in response to growth factors or E_2 was observed when cells were transfected with the ΔMTV-LUC plasmid reporter (not shown). For the series of transfection studies conducted here with ΔMTV-ERE-LUC reporter, ICI 182780 added alone reduced the expression of luciferase to between 65 and 10% of untreated control values; in most cases this was to the level of luciferase activity detected after transfection of cells with the ΔMTV-LUC plasmid reporter. Typically, where the degree of basal inhibition was large, E_2 or IGF-1 gave a poor induction of luciferase.

Table 1. The effect of estradiol and ICI 182780 treatment on cell numbers and prolactin synthesis by GH_3 cells

Treatment	Cell number per well × 10^{-5}	Prolactin (ng/ml)
Control	4.5 ± 0.1	52 ± 7
E_2 10^{-13}	4.4 ± 0.2	68 ± 17
10^{-12}	4.6 ± 0.2	75 ± 17
10^{-11}	4.6 ± 0.3	308 ± 63
10^{-10}	4.8 ± 0.2	595 ± 42
10^{-9}	4.5 ± 0.1	453 ± 105
ICI 10^{-8}	3.3 ± 0.3	ND
ICI (10^{-8}) + E_2(10^{-10})	3.5 ± 0.1	ND

All treatments are molar concentrations and the values given represent mean ± SD (n = 4). ND = not detectable.

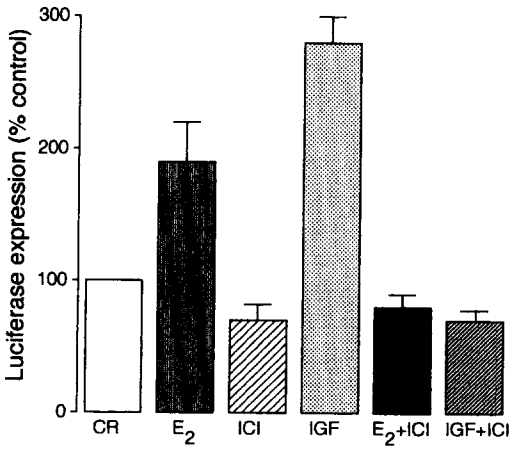

Fig. 4. Luciferase expression by GH_3 cells transfected with the ΔMTV-ERE-LUC reporter plasmid and β-galactosidase expression vector in response to treatments indicated. Luciferase activity was corrected for transfection efficiencies and data points are expressed as percent control for mean values (n = 3), with error bars as CV for the untransformed data. Treatments were added to transfected cells to give final concentrations of the following: E_2, 1 nM; ICI 182780, 10 nM; IGF-1, 10 nM.

DISCUSSION

A considerable body of evidence is now accumulating for a cross-talk between peptide and steroid pathways [21]. Indeed for some cell systems described previously in this report, growth factors appear to be essential for the mitogenic effects of estrogens. On the other hand, where the growth response to E_2 is lost, cell growth can still be inhibited by antiestrogens [22]. Our early findings with the pituitary cell line GH_3, were similar to those of the latter study and indicated that the ER was involved in the growth of these cells but not in response to E_2. These cells were therefore chosen to test the hypothesis that the ER was a potential nuclear factor mediating growth factor induced mitogenesis. Our studies presented here strongly suggest that even in the absence of estrogen, the ER is essential for the mitogenic effects of insulin, IGF-1 and as yet, unidentified autocrine factors produced by these GH_3 cells. We make the claim that the ER is without any known ligand, as extensive experiments (data not shown) were conducted to excluded the presence of estrogens in the medium used for these experiments.

Since growth is a complex process and very few of the genes involved have been characterized, it is possible that in the absence of a growth response to E_2, the antiestrogen-liganded receptor interferes in a non-specific way with the transcription of an essential growth promoting gene. Our data on the secretion of prolactin, the gene for which ER is regulated [20], strongly implies that the above is not the case for this gene. Our transfection data, discussed below, may also be cited as further support against a non-ERE mediated effect of antiestrogen. Also, the hypothesis of interference of the antiestrogen-liganded receptor with transcription of essential growth promoting gene(s) by

peptide factors ultimately acting at a further 5′ responsive element, only holds if growth of the cells is truly estrogen unresponsive. We have recently shown that when cells are transferred from the stock condition to phenol red free DMEM or Hams F12/DMEM with 5% SFCS for one passage prior to seeding and treatment, a mitogenic response to E_2 is observed at concentrations as low as 1 pM (unpublished data).

Although it is still possible for other cellular systems that the ER is essential for a transcriptional response via a DNA element other than the ERE, our transfection data strongly indicate that for GH_3 cells, the growth response to insulin and IGF-1 is mediated via the ERE. Therefore it would appear that the activation of growth factor pathways results in the conversion of the ER to a transcriptionally active state. In effect therefore, under circumstances where estrogen concentrations are low or these compounds are completely absent, the ER in these cells is the nuclear component transducing the growth factor signal. That estrogen fails to give a mitogenic response under the growth conditions employed here, is consistent with our observations from transfection studies that under some conditions, E_2 is a poor inducer of the reporter gene. Therefore it would appear that even the endogenous growth inducing peptides are sufficient to activate transcription driven by the powerful palindromic vitellogenin ERE of the ΔMTV-ERE-LUC construct. As indicated above and supported by others [23, 24] growth can be exquisitely sensitive to estrogen, therefore our data for GH_3 cells, indicates that the transactivating activity of the ER induced by peptide factors, is alone sufficient for a maximum growth response via the ER pathway. It is likely that the loss of estrogen sensitivity, on removal of serum and estrogen, results from the upregulation of autocrine growth factor synthesis or that of their receptors. In this regard, it has already been established that these cells secrete IGF-1 [25]. In contrast to growth, our data would suggest that for prolactin, induction of synthesis can only be achieved by E_2 acting in synergism with autocrine/paracrine factors. It has been observed that the ERE of the rat prolactin gene is a non-palindromic sequence [26]. Where such an imperfect responsive element exists, it has been suggested that the influence of other transcription factors, at adjacent sites, is required for the activation of the promoter sequence [27]. This is indeed the case for the rat prolactin gene since the pituitary specific transcription factor, Pit-1, is required for its full activation [20]. In view of this, it is highly likely that the interaction between Pit-1 and the ER requires the latter to be liganded by estrogen. These data therefore suggest a marked promoter dependence for the transactivating potential of the ER activated by growth factors alone, in comparison to estrogen and peptide factors together—an idea now supported for the synergistic activation of the ER by E_2 and protein kinase activators [28].

During the preparation of this manuscript data has been presented for two *in vitro* studies [29, 30] suggesting that the mechanism of transcriptional activation of the ER reported here, is applicable to other cellular systems. By extrapolation, it is possible that a non-ligand binding form of the ER is used by growth factor pathways in cell types where the ER is not detected by conventional biochemical or immunological methods. It is perhaps by the upregulation of a non-ligand binding form of the ER that some breast tumours progress from an estrogen sensitive, to an estrogen and antiestrogen insensitive state. Recently it has been demonstrated that a non-ligand binding form of ER exists in meningioma [31]. It remains to be established whether this aberrant receptor, possibly constitutively activated by growth factor pathways, is responsible for the high levels of progesterone receptor often observed in these tumours [32].

This study has provided new information as to the nuclear events that occur in response to growth factor binding to cell surface receptors and also raises fundamental questions as to the way steroid receptors can be converted to a transcriptionally active state. In terms of the clinical situation, because of dietary intake, it is clearly not possible to exclude every source of estrogen, even with the powerful aromatase inhibitors now available. These data strongly suggest that even if this was possible, the ER can still function as a transcription factor involved in growth. For this reason, careful regard should be paid to this observation when future strategies are being designed for the treatment of tumours whose growth are sensitive to the 'transcriptionally active ER'.

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