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Cross-Talk between Peptide Growth Factor and Estrogen Receptor Signaling Pathways

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ABSTRACT

The classical receptor for estradiol is a member of a superfamily of nuclear receptors that function as hormone-regulated transcription factors. The ability of the estrogen receptor (ER)-α to activate target gene transcription is mediated by two transcriptional activation functions (AF): AF-1 located in the amino-terminal domain and AF-2 found in the carboxyl-terminal portion of the molecule. The ligand binding domain overlaps AF-2, and upon estrogen binding this region undergoes a conformational change that enables it to contribute to the receptor's transcriptional activity. ER activation is accompanied by increased phosphorylation, and in the absence of ligand, activators of protein kinase A or inhibitors of protein phosphatases are able to stimulate ER-dependent gene expression. More importantly, polypeptide growth factors, such as epidermal growth factor (EGF) and insulin-like growth factor-I (IGF-I), also stimulate the ER's transcriptional activity in an estrogen-independent manner. The AF-1 domain appears to be required for activation by EGF and IGF-I, and point mutation of a single phosphorylation site located within this domain inhibits the ability of growth factor to activate the ER. Thus, steroid receptor function may be regulated by estrogenic ligands as well as by pathway "cross-talk" from membrane receptors for growth factors.

ESTROGEN RECEPTOR STRUCTURE AND FUNCTION

The estrogen receptor (ER) belongs to a nuclear receptor superfamily of ligand-regulatable transcription factors. The ER is a modular protein consisting of an amino-terminal region (A/B domain) that influences transcriptional activity, a centrally located DNA binding region (C domain), and the carboxyl-terminal region (domain E), which enables the receptor to bind to its ligand, estradiol [1]. The hinge region (domain D) is located between the DNA and hormone binding domains, while the F domain is located at the extreme carboxyl terminus of the molecule [1]. It is well established that estradiol binding to the ER, which is accompanied by a conformational change in the hormone binding domain [2, 3], results in the activation of this receptor and increased transcription of ER target genes. Two distinct regions within the ER contribute to its transcriptional activity: the constitutively active, amino-terminal activation function (AF-1) and the ligand-regulatable AF-2 domain, which is located in the carboxyl-terminal portion of the molecule (Fig. 1). Depending on the cell type and promoter examined, AF-1 and AF-2 may regulate transcription independently or synergistically [4, 5]. Although the F domain is not required for hormone binding, it does modulate transcriptional activity [6, 7].

It is generally accepted that site-specific transcription factors, such as steroid receptors, stimulate gene expression by promoting the assembly of basal transcription factors (TF) into a stable preinitiation complex that facilitates an increased rate of transcription initiation by RNA polymerase II [8, 9]. Although the ER can interact directly with TFIIB [10], positively acting factors, termed coactivators, are envisioned to serve as bridging factors between specific activators and general transcription factors [11–14]. Although many hormone-dependent, ER-interacting proteins have been identified, only a subset consisting of SRC-1, TIF2 (and its murine homolog, GRIP1), p/CIP (also known as ACTR, RAC3, or AIB1) and CBP significantly stimulate (≥ 3-fold) ER-mediated transcription [15–28]. Notably, SRC-1, TIF2, and p/CIP share extensive homology, and it is likely that they comprise a family of steroid receptor coactivators [23, 27]. In addition to the ability of CBP to synergize with SRC-1 in the activation of steroid receptor-dependent transcription [21], CBP also serves as a coactivator for a diverse array of transcription factors including c-Myb [29], c-Fos [30], and c-Jun [31], and in this role it appears to serve as an integrator protein [32].

ER PHOSPHORYLATION

Activation of the ER by estrogenic ligands is associated with increases in overall receptor phosphorylation [33–39]. Using a combination of direct (amino acid and radiolabel sequencing) and indirect (deletion and site-directed mutagenesis) approaches, five phosphorylation sites have been mapped within the human ER (Fig. 1). Four of these sites (serine^{104}, serine^{106}, serine^{118}, and serine^{167}) are located in the A/B domain while the fifth site (tyrosine^{537}) is located within the ligand binding domain [35, 37, 38, 40]. The phosphorylation of the amino-terminal serine sites is hormone-inducible, and it has been proposed by several laboratory groups that serine^{118} is the major estrogen-inducible phosphorylation site in ER isolated from COS-1 cells, while another group has reported that serine^{167} fulfills this role in MCF-7 cells [35, 37, 38]. Mutation of three of the amino-terminal phosphorylation sites (serine^{104}, serine^{106}, and serine^{118}) to alanine residues significantly reduces ER transcriptional activity and highlights the importance of these amino acids for full receptor function [35, 37]. An explanation for the presence of two distinct, major hormone-inducible phosphorylation sites has not been established, but it is possible that phosphoacceptor site usage may be related to the different cell types used in these studies. In support of this, it has been demonstrated that the extent to which phosphorylation mutants affect ER transcriptional activity varies with both cell type and promoter context. For example, a point mutant (serine^{118} → alanine^{118}) form of human ER is more active in COS-1 than in HeLa cells and is a more effective activator of the pS2-CAT synthetic target gene than the chicken ovalbumin gene-derived reporter construct, pDH3OV/CAT, in both cell lines [35]. One possible explanation for these cell- and promoter-dependent differences in ER mutant transcriptional activity is that receptor-mediated gene expression is influenced by cell- and/or promoter-specific accessory proteins, such as coactivators and corepressors [24, 41].

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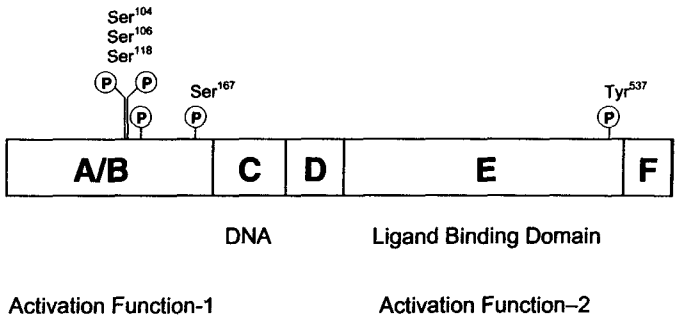

FIG. 1. Full-length human ER is composed of 595 amino acids. The location of the DNA (domain C) and ligand binding (domain E) regions are indicated. The position of the amino-terminal (Activation Function-1) and carboxyl-terminal (Activation Function-2) activation domains also are shown. The position of the five known phosphorylation sites in human ER are represented by "P." (Ser, serine; Tyr, tyrosine).

Estrogens do not induce phosphorylation of tyrosine^{537}, and this amino acid is therefore referred to as a basal phosphorylation site [40]. This residue is conserved in the ER sequence of every species examined to date and is located immediately amino-terminal to the AF-2 activation helix [42]. This amphipathic, α-helical structure is critical for AF-2 function, and mutagenesis studies suggest that coactivator interactions with the ER may depend on the integrity of this activation region [16, 43]. The importance of this tyrosine (tyrosine^{537} in human ER, tyrosine^{541} in mouse ER) for ER function is highlighted by site-directed mutagenesis studies in which amino acid substitutions (serine or alanine for tyrosine^{537} in human ER; aspartic acid, glutamic acid, or alanine for tyrosine^{541} in mouse ER) create constitutively active ER mutants [42, 44]. In contrast to wild-type ER, these receptor mutants interact with coactivator proteins such as SRC-1, RIP140, TIF1, and to a lesser extent ERAP140 in the absence of hormone [42, 44, 45]. Taken together, these data suggest that tyrosine^{537} and the adjacent amphipathic helix form a conformation-dependent interaction surface for coactivators, and that this region is important for ER transcriptional activity.

SIMILARITIES BETWEEN GROWTH FACTOR AND ESTROGEN RESPONSES

Estrogens, acting via the ER, are important regulators of the growth, differentiation, and function of many reproductive tissues including the uterus, vagina, ovary, mammary gland, and brain. The ability of estrogens to stimulate cell proliferation is well established and can be characterized experimentally by increases in DNA synthesis, as well as by changes in cell morphology. In the uterus and mammary gland, estrogen-induced proliferation is accompanied by increased mRNA and protein expression of polypeptide growth factors (epidermal growth factor [EGF], transforming growth factor α [TGFα], and insulin-like growth factor-1 [IGF-I]) and their receptors, and it has been suggested that these growth factors may act as autocrine and/or paracrine mediators of estrogen action [46–55].

A role for growth factor signaling in the estrogenic regulation of uterine biology was demonstrated in a series of experiments done by Korach and McLachlan [56, 57]. Administration of EGF antibodies to ovariectomized mice 3 days before hormone treatment significantly reduced the ability of estradiol to induce DNA synthesis and suggested that EGF may mediate, in part, the proliferative effects of this steroid ligand. When EGF was administered via slow-release pellets implanted under the kidney capsule, uterine and vaginal epithelial cell proliferation was stimulated, further supporting the hypothesis that EGF could mimic estrogen-like proliferative responses. These experiments were done in hypophysectomized, adrenalectomized, and ovariectomized mice, indicating that neither ovarian, adrenal, or pituitary hormones were required to stimulate cell proliferation in EGF-treated animals. The subsequent demonstration that the pure antiestrogen ICI 164,384 could partially block EGF-induced, uterine DNA synthesis indicated that at least a portion of the proliferative response was mediated by the ER and suggested that EGF signaling pathways could cross-talk with the ER [57].

Growth factors also mimic estrogens in their ability to increase the expression of ER target genes, such as the progesterone receptor (PR) and the iron-binding glycoprotein, lactoferrin. Early experiments demonstrated that treatment of MCF-7 breast cancer or primary uterine cells with IGF-I or EGF increased PR binding activity [58–60]. Cotreatment with either of the ER antagonists LY 117,018 or ICI 164,384 blocked growth factor as well as estradiol induction of PR expression, suggesting that both stimuli could activate ER function. In animals treated with EGF, lactoferrin expression is elevated in uterine epithelial cells [56]. This increased expression of this gene is similar to that observed in mice treated with the synthetic estrogen diethylstilbestrol [56].

More recently, experiments done with the ERα knockout (ERKO) mouse also support the hypothesis that some of the cellular effects of EGF are mediated via the ER. Both alleles of the ERα gene have been disrupted by homologous recombination in the ERKO mouse [61], and a preliminary report indicates that ERβ expression patterns do not appear to be significantly altered in these animals [62]. Expression of ERβ is not detectable in mouse uterus [62], and estrogenic responses in this organ are presumably the result of ERα activity. EGF treatment of ERKO mice does not increase uterine DNA synthesis as it does in wild type animals, nor can it induce PR mRNA expression [63]. The inability of EGF to elicit these proliferative and transcriptional effects in the ERKO mouse is not due to a general defect in EGF signaling [63]. Wild-type and ERKO mice express comparable levels of EGF-receptor in the uterus, which autophosphorylates in response to EGF challenge. Furthermore, induction of the EGF target gene, c-*fos*, is robust in wild-type and knock-out mouse uterine tissue. Taken together, these data indicate that EGF exerts some of its biological responses in an ER-dependent manner and, therefore, provides evidence that growth factor signaling pathway(s) can cross-talk with, and regulate, the activity of the ER in vivo.

The ability of ER antagonists to block some of the cellular actions of growth factors suggested that this nuclear receptor may be capable of receiving direct inputs from signal transduction pathways. Several experiments suggest that EGF stimulation can alter some of the ER's biochemical properties. It is well established that estrogen treatment increases the proportion of ER in the nucleus in comparison to the cytoplasm and enhances the receptor's affinity for chromatin [64]. In cells treated with EGF, most ER is found in the nuclear compartment [57]. In addition, this nuclear ER appears as heterogenous bands by Western blot analysis, just as receptor obtained from estrogen-treated cells also yields multiple bands [57]. Alterations in ER mobility assessed by this method are associated with increased re-

ceptor phosphorylation [34, 65], which suggests that the ER may be a direct target of growth factor signaling pathways.

ER LIGAND-INDEPENDENT ACTIVATION

Mechanistic studies have defined several molecular events associated with estrogen-dependent activation of the ER, including changes in receptor conformation [2, 66], increased interaction with coactivators [16, 18, 32, 45), increased receptor phosphorylation [36–38], and alterations in the ability of receptor to bend DNA [45, 67]. Although the mechanism(s) by which steroid ligands increase receptor phosphorylation are poorly defined at the present time, ligand-dependent activation of all steroid receptors studied to date, including the ER, is accompanied by an increase in receptor phosphorylation [68]. The correlation between receptor phosphorylation and transcriptional activity suggests that these two events are functionally linked.

In the first transient transfection studies demonstrating ligand-independent activation of a steroid receptor, 8-bromo-cAMP (an activator of protein kinase A) or okadaic acid (an inhibitor of protein phosphatases 1 and 2A) was used to activate chicken PR-dependent expression of a synthetic target gene [69]. Similarly, human ER can be ligand-independently activated by okadaic acid in CV_1 (green monkey kidney) cells, and rat ER is transcriptionally activated by cholera toxin and 3-isobutyl-1-methylxanthine (IBMX, a phosphodiesterase inhibitor), or 8-bromo-cAMP in primary rat uterine cell cultures [36, 70]. In other cell types (MCF-7 and Chinese hamster ovary cells), neither cholera toxin/IBMX nor 12-*O*-tetradecanoylphorbol 13-acetate (TPA; an activator of protein kinase C) were able to increase ER-dependent reporter gene expression in the absence of estradiol [71]. The variability in the ability of phosphorylating agents to modulate ER activity in a ligand-independent manner is likely to reflect promoter or cell-type specific differences in ER function. These differences also have been noted to influence basal [72] and estrogen-stimulated ER-dependent transcription [73]. Indeed, the ability of TPA to synergize with estradiol in the activation of ER-dependent transcription varies with these two parameters [71].

Studies with pharmacological agents established the potential of the ER to be activated in the absence of estrogen. The subsequent demonstration that several naturally occurring substances, including the neurotransmitter dopamine [74] and growth factors (see below), could stimulate ER transcriptional activity in transient transfection experiments firmly established the ability of the ER to respond to biologically relevant, ligand-independent signaling pathways. The transcriptional activity of the ER can be stimulated in cells treated with EGF, although to a lesser extent than in cells treated with physiological concentrations of estradiol [75–77]. Furthermore, the ability of EGF to stimulate gene expression requires the presence of both the ER and an estrogen response element in the promoter region of the synthetic target gene [75]. Taken together with the ability of the pure antiestrogen ICI 164 384 to block EGF activation of gene expression [75, 77], these data confirm that EGF signaling pathway(s) can cross-talk with the ER and modulate the activity of this transcription factor. Neutralizing antibodies to the EGF receptor block ER-dependent gene expression stimulated by EGF [75] and indicate that this growth factor must act through its plasma membrane receptor to communicate with the ER. Another mitogen, TGFα, which also binds and activates the EGF receptor, stimulates ER transcriptional activity in a similar fashion [75].

In addition to EGF and TGFα, several other growth factors are capable of activating ER-dependent gene expression. In primary rat uterine cell cultures treated with IGF-I, target gene expression is stimulated to an extent similar to estradiol, and both responses were blocked by ICI 164 384 as well as the cyclic nucleotide-dependent protein kinase inhibitor, H8 [36]. IGF-I also activates the ER in GH_3 pituitary cells [78] and the neuroblastoma cell line, SK-ER3, where, like estradiol, it inhibits cell growth and promotes differentiation [79]. Insulin also will stimulate ER activity in neuroblastoma cells in a ligand-independent manner [79, 80]. In addition, the EGF-like growth factor, heregulin, acting through erb B2/erb B3 heterodimeric receptors in MCF-7 cells, appears to stimulate proliferation and PR gene expression in an ER-dependent manner [81]. Thus, in diverse cell types, a variety of growth factors can initiate signaling pathways that cross-talk with the ER, and it therefore appears likely that ligand-independent activation of the ER is not a cell-restricted event.

The structural features of the ER required for its activation by growth factor signal transduction pathways are incompletely understood. Human or mouse ER mutants lacking the amino-terminal, AF-1 domain are activated by estradiol, but not by EGF or IGF-I signaling pathways [75, 76, 82]. Conversely, ER mutants lacking the ligand binding domain are activated by EGF and IGF-I, but not by estradiol, and this indicates that the AF-1 domain is necessary to respond to growth factor signaling pathways [75, 76, 82]. In contrast, insulin activation of the ER does not require the A/B domain, and receptor deletion mutants consisting of the DNA and ligand binding domains are active in insulin-treated cells to nearly the same extent as they would be in estrogen-treated cells [80]. Co-administration of estradiol and EGF or IGF-I increases ER target gene expression to an extent greater than either agent alone, and in some instances, this response appears to be synergistic [82]. Interestingly, the ability of IGF-I and estrogen-dependent activation pathways to synergize does not require the AF-1 domain [82], suggesting that growth factor signaling pathways may target different regions of ER depending on the presence or absence of ligand, potentially as a result of the different receptor conformations induced by ligand.

Treatment of primary rat uterine cells with IGF-I increases the overall extent of ER phosphorylation [36]. Similarly, ER phosphorylation in COS-1 and SK-Br-3 breast cancer cells is stimulated by EGF and IGF-I to an extent similar to that by estrogen [77, 83]. EGF is unable to stimulate the phosphorylation of an alanine^{118} mutant of full-length or carboxyl-terminal-deleted ER [77, 83], and this is consistent with the A/B domain's being critical for ER activation by EGF. The transcriptional activity of an alanine^{118} ER mutant examined in COS-1, HeLa, or SK-Br-3 cells is not increased by EGF or by expression of a dominant active Ras mutant [77, 83]. Interestingly, when a glutamic acid is substituted for serine^{118}, the receptor retains its ability to respond to EGF signaling pathways, suggesting that a negative charge at position 118 may contribute to growth factor activation of ER transcriptional activity [77].

GROWTH FACTOR SIGNALING PATHWAYS CROSS-TALK WITH THE ER

The plasma membrane-associated receptors for EGF, TGFα, heregulin, IGF-I, and insulin are type I tyrosine ki-

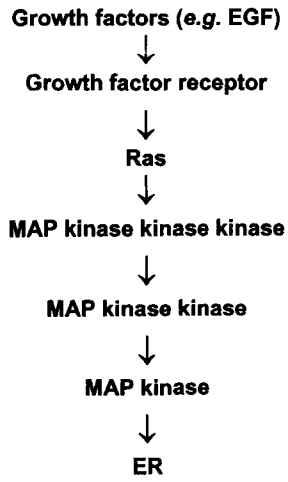

FIG. 2. Schematic representation of an MAPK signaling pathway. Binding of growth factors to their cognate receptors results in the intracellular activation of a MAPK signal transduction cascade that ultimately influences the transcriptional activity of the ER.

nase receptors. Upon binding of a growth factor to the extracellular domain of its receptor, it undergoes autophosphorylation on tyrosine residues and subsequently acquires the potential to activate a number of intracellular enzymatic activities. One intracellular pathway activated by EGF binding to its receptor is the mitogen-activated protein kinase (MAPK) signal transduction cascade (Fig. 2). Briefly, growth factor receptor activation enables the docking protein, Grb2, via its SH2 domain, to bind to phosphotyrosine residues located in the cytoplasmic portion of the receptor, and this protein in turn recruits the nucleotide exchange factor SOS and then Ras to the intracellular surface of the plasma membrane [84]. In the GTP-bound, active state, Ras interacts with and promotes the activation of MAPK kinase kinases, such as Raf-1. These enzymes are serine kinases that phosphorylate and thereby activate MAPK kinases, such as Meks 1 and 2, which in turn activate MAPKs, such as Erks 1 and 2, through phosphorylation of threonine and tyrosine residues [85]. Once activated, MAPKs phosphorylate target proteins, including transcription factors, such as c-Jun or ATF-2. Recent studies indicate that there are three distinct types of MAPK signaling pathways that can be activated by mitogens, extracellular stresses such as ultraviolet radiation, and hyperosmolarity; but to date, only growth factors have been shown to modulate ER function.

Several approaches have been employed to establish that an EGF-induced MAPK signaling cascade can activate the ER. Overexpression of a dominant active Ras mutant, c-Ki-Ras^{Val12}, increases the transcriptional activity of ER mutants lacking the ligand binding domain, whereas receptor constructs lacking the A/B domain are not influenced by this Ras mutant [83]. Furthermore, the activity of an alanine^{118} ER mutant is not enhanced by this constitutively active form of Ras [83]. In contrast, the dominant-negative Ras mutant, RasN17, attenuates the ability of EGF to activate ER target gene expression [77]. Expression of a dominant active form of MAPK kinase increases phosphorylation of a ER deletion mutant consisting of the A/B and DNA binding domains, but not the corresponding receptor in which serine^{118} is replaced by an alanine residue [83]. Correspondingly, expression of a dominant-negative MAPK mutant suppressed EGF-induced, ER transcriptional activity [77]. Lastly, purified MAPK phosphorylates the amino-terminus of ER in vitro, but not when serine^{118} is replaced by either an alanine or glutamic acid residue [83]. Thus, constitutively active components of the MAPK signal transduction pathway can regulate ER transcriptional activity independently of EGF, and dominant negative forms of the MAPK cascade inhibit EGF activation of the ER. Taken together, these data strongly suggest that the MAPK signal transduction cascade is responsible for growth factor activation of the ER.

PHYSIOLOGICAL SIGNIFICANCE OF GROWTH FACTOR CROSS-TALK

The importance of growth factor induction of ER transcriptional activity to normal physiological responses is not clear at the present time. It is possible that growth factors maintain moderate levels of ER transcriptional activity when estrogen levels are very low, such as in males or in females during proestrous. Alternatively, signaling cascades initiated by growth factors binding to their receptors may modulate the transcriptional activity of ligand-occupied ER and thereby increase the magnitude of target gene expression. It is not known whether the target genes activated by ER in response to growth factors and estrogens are identical, or whether there are genes that are preferentially or selectively regulated by one or the other pathways. In the context of the ability of estrogens to increase the expression of some growth factors and their receptors, it is an intriguing possibility that cross-talk between growth factor and steroid hormone signaling pathways may establish a positive feed-back system. Low levels of estrogen could increase growth factor expression, and together these stimuli may promote a stronger or more sustained biological response in target tissues. It is also conceivable that cross-talk pathways may sensitize the ER to suboptimal stimulation by low levels of estrogens and thereby promote biologically meaningful responses under conditions in which ligand stimulus alone would be unable to generate a significant signal.

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