Estrogen Receptor Phosphorylation

HORMONAL DEPENDENCE AND CONSEQUENCE ON SPECIFIC DNA BINDING*

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We have shown that the 32P-phosphorylation of the nuclear estrogen receptor from human MCF-7 cells or the calf uterus is estrogen-dependent. Within 2 min of estradiol treatment the phosphorylation of the estrogen receptor from MCF-7 cells doubled, and increased 4-fold within 20-40 min of estradiol treatment. Progesterone was ineffective in stimulating the phosphorylation of the estrogen receptor. Phosphoamino acid analysis indicated that the estrogen-stimulated phosphorylation of the human or calf estrogen receptor occurred only on serine residue(s). Phosphotryptic peptide analysis of the human estrogen receptor by two-dimensional peptide mapping or reverse-phase high performance liquid chromatography revealed that only a single tryptic peptide (site) was phosphorylated. Treatment of the estrogen receptor with potato acid phosphatase resulted in the dephosphorylation of the 32P-labeled estrogen receptor and a decrease of the receptor's affinity for specific DNA sequences. These data suggest that transcriptional activation by the estrogen receptor involves an estrogen-dependent phosphorylation of the receptor resulting in its increased affinity for specific DNA sequences.

The estrogen receptor is a transcriptional regulatory protein that is allosterically regulated by estrogen binding (Notides et al., 1981; Sasson and Notides, 1988). The estrogen-receptor complex interacts with specific nucleotide sequences termed "estrogen-responsive elements" (ERE) that are located in the 5'-flanking region of responsive genes (Walker et al., 1984). It has been shown, using gene transfer experiments that the ERE, estrogen receptor, and hormone are sufficient to confer estrogen responsiveness to a cell (Maurer and Notides, 1987; Burch et al., 1988; Berry et al., 1989).

There is growing evidence that phosphorylation/dephosphorylation is involved in the modulation of the activity of such trans-acting factors as SP-1 (Jackson et al., 1989), SRF (Frywes et al., 1988), glnALG (Magasanik, 1989), ADR 1 (Taylor and Young, 1989), CAMP response element-binding protein (Lamph et al., 1990), and Pit 1 (Kaploft et al., 1991). Site-directed mutagenesis and gene transfer studies of CAMP response element-binding protein and glnALG have indicated the importance of phosphorylation at specific amino acid residues in the regulation of their transcriptional activity (Lee et al., 1990). Interestingly, many steroid hormone receptors have been shown to be phosphorylated proteins. In intact cells, the progesterone (Sullivan et al., 1988; Sheridan et al., 1989), glucocorticoid (Munch and Brink-Johnson, 1968; Housley and Pratt, 1983), and vitamin D (Pike and Sletor, 1985; Jones et al., 1991) receptors are phosphorylated after exposure to their cognate ligands. However, the exact biochemical consequence of the ligand-induced phosphorylation remains to be defined. Investigators have attempted to causally link the phosphorylation status of the steroid hormone receptors with hormone binding (Mendel et al., 1986), DNA binding (Tienrungroj et al., 1987), and receptor turnover and processing (Horwitz and McGuire, 1978). Sheridan et al. (1988) have shown that phosphorylation of the progesterone receptor is a nuclear event and have suggested that it may be involved with transcriptional activation of progesterone-regulated genes. Aurrichio and co-workers (Migliaccio et al., 1989) have reported that the estrogen receptor is phosphorylated on tyrosine residues in response to estradiol treatment, and that phosphorylation of the estrogen receptor is related to the ability of the estrogen receptor to bind hormone.

Here we report the action of estradiol on the kinetics and characteristics of phosphorylation of the estrogen receptor in the MCF-7 breast adenocarcinoma cell line using a highly specific antibody against the estrogen receptor. Our evidence strongly indicates that an estrogen-dependent phosphorylation of a specific serine residue(s) of the estrogen receptor enhances its binding to specific DNA sequences.

EXPERIMENTAL PROCEDURES

Labeling of the Estrogen Receptor in MCF-7 Cells and Calf Uterine Slices with 32P-Phosphate—Confluent MCF-7 cells (100-mm plates) or finely minced calf uteri (100 mg/ml) were fed with serum-free low phosphate (10 μM KH2PO4) minimal essential medium containing 250 μCi of [32P]orthophosphate/mll for 4 h at 37 °C, then treated with 50 nM estradiol for 5–120 min followed by rinsing with ice-cold Hanks' balanced salt solution. The cells were homogenized in 1 ml of homogenization buffer (10 mM HEPES, pH 7.4, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1 mM EDTA, 0.25 mM EGTA, 0.5 mM leupeptin, and 0.2 mM phenylmethylsulfonyl fluoride) containing 0.5 M NaCl at 0 °C and centrifuged for 1 h at 100,000 × g. The estrogen receptor was immunopurified by incubation of the cytosol with the anti-calf estrogen receptor antibody for 3 h at 0 °C and then adsorbed to Protein A using 200 μl of a 50% Protein A-Sepharose slurry (Pharmacia LKB Biotechnology Inc.). The Protein A-Sepharose/immunocomplexed receptor was washed...
with 40 mM Tris, pH 7.4, 0.5 M NaCl, and 0.05% Triton X-100. The estrogen receptor was eluted from the Protein A-Sepharose by boiling in Laemmli sample buffer and subjected to SDS-gel electrophoresis at 50 mA/gel and visualized by autoradiography (Laemmli, 1970). For phosphomonoacid analysis the 32P-phosphorylated estrogen receptor was excised from the dried gel. The gel was swollen, homogenized, and calf-reduced with 50 mM NH4CO3 and 10% β-mercaptoethanol for 18 h at 65 °C. The protein was precipitated with 10% trichloroacetic acid then partially hydrolyzed with 6 M HCl for 2 h at 100 °C. The phosphomono acids were detected as described by Hunter and Selton (1980).

Analysis of the Tryptic Peptides of the 32P-Phosphorylated Estrogen Receptor—The immunopurified 32P-estrogen receptor was subjected to SDS-gel electrophoresis and autoradiography. The receptor was electroeluted from the gel and its sulfhydryl bonds oxidized with performic acid. Then the receptor was digested with 1 μg of trypsin for 16 h followed by an additional 1 μg of trypsin for 5 h at 21 °C. The digest was loaded onto a 250-μm thin layer Avicel plate and subjected to electrophoresis at 500 V for 1 h in 50 mM NH4HCO3, pH 8.9. The plate was then dried and the peptides resolved chromatographically in the second dimension using pyridine:acetic acid:water (1:10:189), pH 3.5. The [32P]phosphate-labeled tryptic peptides of the estrogen receptor were separated by HPLC with a C8 reverse-phase 300 column (Applied Biosystems). The peptides were eluted using a 0–100% acetonitrile gradient containing 0.1% trifluoroacetic acid for 16 h followed by an additional 1 μg of trypsin for 24 h at 21 °C. The eluant was monitored at 215 nm.

Anti-estrogen Receptor Antibody Production—Approximately 200 μg of the calf uterus estrogen receptor were separated from the calf uterus by estradiol and heparin affinity chromatography (Maurer and Notides, 1987) was used to immunize a female New Zealand White rabbit. The estrogen receptor (100 μg) in complete Freund's adjuvant was injected subcutaneously as multiple doses over a 1-month period. After 3 weeks, 100 μg of receptor in incomplete Freund's adjuvant was injected subcutaneously as multiple doses over a 2-month period. The rabbit was bled and its sera showed a 50% immunoprecipitation of the calf or rat [3H]estradiol-receptor complex at a 1:400 dilution; human estrogen receptor showed a 50% immunoprecipitation with a 1:200 dilution of the sera.

Estrogen Receptor Phosphorylation—The phosphorylation of the estrogen receptor was strongly dependent upon estradiol binding. The MCF-7 cellular [32P]ATP pool reached isotopic equilibrium within 4 h of incubation with [32P]orthophosphate (data not shown). Under these conditions, very little [32P]phosphate labeling of the estrogen receptor was observed in MCF-7 cells that were not exposed to estradiol (Fig. 1, lane 1). When an equal number of 32P-labeled MCF-7 cells were treated with 50 nM estradiol for 40 min, the estrogen receptor was observed to have incorporated a much greater amount of [32P]phosphate than control cells (Fig. 1, lane 2). Exposure of the MCF-7 cells to 100 nM progesterone for 40 min did not result in phosphorylation of the estrogen receptor (Fig. 1, lane 3), demonstrating that the phosphorylation of the receptor is estrogen-dependent. Preimmune serum did not immunoprecipitate the 32P-labeled estrogen receptor (Fig. 1, lane 4). An immunoblot of the cellular proteins from MCF-7 cells showed that the rabbit anti-calf estrogen receptor antibody recognized the human estrogen receptor (Fig. 1, lane 5). In addition, sucrose gradient analysis showed that the anti-calf estrogen receptor antibody recognized and formed a complex with the native 4 S, 5 S, or 8 S [3H]estradiol-receptor complexes from calf or human but did not recognize the calf progesterone or glucocorticoid receptor (data not shown). The estrogen receptor from the calf uterus was also phosphorylated in response to estradiol treatment (Fig. 2, compare lanes 1 and 2). The basal level of 32P-labeled-estradiol receptor in calf uterine slices was considerably higher than the basal level of receptor phosphorylation in MCF-7 cells (compare lanes 1 in Figs. 1 and 2). Thus, the phosphorylation of the estrogen receptor was increased with estradiol treatment in human and bovine cells.

The rate of estrogen receptor phosphorylation was rapid, closely paralleling the nuclear retention of the receptor (Fig. 3). This suggested that steroid and nuclear binding may be a prerequisite for estrogen receptor phosphorylation. Within 2 min of 50 nM estradiol exposure, the phosphorylation of the

![Fig. 1. Phosphorylation of the human estrogen receptor in MCF-7 cells. The estrogen receptor was labeled with [32P]orthophosphate, immunopurified with the anti-calf estrogen receptor antibody, and analyzed by SDS-gel electrophoresis followed by autoradiography. The MCF-7 cells were: lane 1, not treated; lane 2, treated with 50 nM estradiol for 40 min; lane 3, treated with 100 nM progesterone for 40 min; lane 4, treated with 50 nM estradiol for 40 min but immunoprecipitated with preimmune serum. An immunoblot of the human MCF-7 cellular extract using the anti-calf estrogen receptor antibody is shown (lane 5).](image-url)
The estrogen receptor was labeled with \[^{32}P\]orthophosphate in calf uterine slices, immunopurified, and analyzed by SDS-gel electrophoresis followed by autoradiography. The uterine slices were: lane 1, not treated; lane 2, treated with estradiol for 40 min; lane 3, treated with estradiol but immunoprecipitated with preimmune serum.

FIG. 2. Phosphorylation of the calf estrogen receptor. The estrogen receptor was labeled with \[^{32}P\]orthophosphate in calf uterine slices, immunopurified, and analyzed by SDS-gel electrophoresis followed by autoradiography. The uterine slices were: lane 1, not treated; lane 2, treated with estradiol for 40 min; lane 3, treated with estradiol but immunoprecipitated with preimmune serum.

The estrogen receptor in the MCF-7 cells also reached a maximal level at 20 min, mirroring the maximal level of receptor phosphorylation (Fig. 3, B and C). Analysis of total estrogen receptor concentration by \[^{3}H\]estradiol binding (Fig. 3C) or immunoblot analysis (data not shown) indicated that the total receptor concentration was unchanged within 2 h of estradiol treatment. Furthermore, the immunoprecipitated receptor from cells not treated with estradiol showed no appreciable change in the phosphate incorporation throughout the duration of the treatment (Fig. 3A, lanes 6 and 7). Therefore, the \[^{3}P\] labeling of the receptor was due to a direct phosphorylation of the nuclear estrogen receptor and not a change in cellular receptor content.

Phosphoamino Acid and Peptide Analysis of the \[^{32}P\]-Labeled Estrogen Receptor—The immune-specific \[^{32}P\]-labeled estrogen receptor from the MCF-7 cells was exclusively phosphorylated on serine residues. Conditions were optimized for liberation and preservation of the phosphorylated amino acids, especially phosphotyrosine. Nevertheless, the only amino acid of the receptor phosphorylated in the MCF-7 cell was serine (Fig. 4). Similarly, only serine residues were phosphorylated on the calf estrogen receptor (data not shown). Two-dimensional peptide mapping of the \[^{32}P\]-phosphate-labeled estrogen receptor showed a single tryptic peptide containing the estrogen-dependent \[^{32}P\]phosphoserine residue(s) (Fig. 5A). The two-dimensional peptide map of the \[^{32}P\]-labeled receptor from MCF-7 cells that were not treated with estradiol was completely devoid of this estradiol-stimulated \[^{32}P\]-labeled peptide (data not shown). Tryptic peptide mapping of the receptor after labeling with \[^{35}S\]methionine resolved nine separate \[^{35}S\]methionine-labeled peptides, indicating that the tryptic digestion and the peptide separation were successful. The tryptic digests of the \[^{32}P\]-labeled receptor were subjected to HPLC analysis using a narrow bore C8 reversed-phase column. Again, a single major \[^{32}P\]-phosphotryptic peptide was observed that eluted at 50 min (at approximately 45% acetonitrile). Two other minor phosphorylated peaks that eluted at 38 and 43 min accounted for 16% of the total \[^{32}P\]phosphate-labeled peptides eluting from the column (Fig. 5B). Thus, the single phosphotryptic peptide of the estrogen receptor stands in contrast to the multiple

FIG. 3. Kinetics of estrogen receptor phosphorylation and \[^{3}H\]estradiol binding in MCF-7 cells. The estrogen receptor was labeled with \[^{32}P\]orthophosphate, immunopurified, and analyzed by SDS-gel electrophoresis followed by autoradiography. The MCF-7 cells were treated with 50 nM estradiol for 2, 10, 20, 45, or 120 min (lanes 1-5, respectively) or without estradiol for 0 (lane 6) or 120 min (lane 7)(A). Densitometric analysis of the \[^{32}P\]-labeled autoradiogram showing the percentage increase in receptor phosphorylation relative to the estrogen receptor from MCF-7 cells that were not stimulated with estradiol (B). Parallel cultures of MCF-7 cells were treated with 50 nM \[^{3}H\]estradiol for 0, 10, 20, and 45 min, and the total (■) and nuclear (○) \[^{3}H\]estradiol receptor concentrations were determined (C).

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FIG. 4. Phosphoamino acid analysis of the estrogen receptor from MCF-7 cells. The estrogen receptor was labeled with \[^{32}P\]orthophosphate, immunopurified, and analyzed by SDS-gel electrophoresis followed by autoradiography. The \[^{32}P\]-labeled receptor was excised from the gel, phosphoamino acid standards added, the receptor hydrolyzed with HCl, then analyzed by thin layer electrophoresis followed by autoradiography. The migration of the phosphoamino acid standards were detected by ninhydrin staining and the migration of the \[^{32}P\]-labeled serine by autoradiography.
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phosphotryptic peptides seen in the glucocorticoid and progestosterone receptors.

Dephosphorylation of the Estrogen Receptor Decreased the Receptor's Avidity for Binding to Specific DNA Sequences—Nuclear extracts prepared from estradiol-treated MCF-7 cells or estrogen receptor purified to homogeneity from calf uterus were incubated with a 32P-labeled 47-base pair DNA fragment containing the perfect estrogen-responsive element of the chicken vitellogenin II gene (Burch et al., 1988). One major (Fig. 6, B2) and two minor (Fig. 6, B1 and B3) protein-DNA complexes were resolved by gel electrophoresis. Protein-DNA complexes with equivalent mobilities were observed with either the nuclear extract of the MCF-7 cells or with the purified calf estrogen receptor (compare lanes 1 and 2 in Fig. 6). The addition of the anti-estrogen receptor antibody to the receptor:32P-labeled DNA mixture eliminated the three bands. This was not observed with the addition of preimmune serum, substantiating that the retarded protein-DNA complexes contained estrogen receptor (Koszewski and Notides, 1991).

Treatment of the estrogen receptor purified from the calf uterus or MCF-7 nuclear extracts with potato acid phosphatase resulted in a loss of the receptor's capacity to bind to the specific DNA sequence (Fig. 6, lanes 3, 4, and 6). However, with the addition of 10 mM phosphate, the acid phosphatase activity was inhibited and consequently the formation of the receptor:32P-labeled DNA complex was unaffected (Fig. 6, lane 7). Immunopurification and SDS-gel electrophoresis analysis showed that the 32P-labeled estrogen receptor from MCF-7 cells was dephosphorylated when treated with the potato acid phosphatase, indicating that the estrogen-dependent phosphorylation site(s) of the receptor was accessible to the phosphatase. In addition, the [3H]estradiol binding capacity of the estrogen receptor was not affected by the phosphatase (data not shown). These data indicate that the loss of the receptor's specific DNA binding activity after treatment with the phosphatase was due to the dephosphorylation of the receptor and not to contaminating proteolytic activity.

DISCUSSION

An understanding of the mechanism by which the estrogen receptor regulates transcription requires identifying all the components that the receptor interacts with to alter the cell's transcriptional machinery. Thereafter, the temporal sequence, nature, and consequence of the modifications that the receptor, transcriptional factors, or DNA undergo to modulate the transcriptional process need to be characterized. Previous molecular analysis of the estrogen receptor demonstrated that the active form of the estrogen receptor is a homodimer (Notides and Nielsen, 1974; Nielsen and Notides, 1975) and that estradiol binding is a positively cooperative-binding mechanism (Weichman and Notides, 1977; Notides et al., 1981) promoting receptor dimerization (Notides et al., 1975) and conformational changes in the receptor that result in an increased affinity for DNA (Skafar and Notides, 1985; Koszewski and Notides, 1991). Recent studies with the recombinant estrogen receptor and its mutant variants have confirmed and extended these characteristics of the estrogen receptor (Kumar and Chambon, 1988; Klein-Hitpass et al., 1989).

In this report we present evidence that following hormone binding, the nuclear estrogen receptor is a target for phosphorylation; the consequences of which influence the receptor's interaction with specific DNA-binding sites, the ERE. We observed that estradiol binding by the estrogen receptor in MCF-7 cells or calf uterine tissue slices is immediately followed by phosphorylation of the receptor. The estrogen-stimulated phosphorylation of the receptor is coincidental with "tight" nuclear binding of the receptor, i.e. buffers con-
Containing 0.4 M salt are required to effectively extract the receptor from the nuclear fraction (Fig. 2). That the estrogen receptor is phosphorylated solely on serine residues in response to estradiol (Fig. 4) is in agreement with Washburn et al. (1991) but is in contrast to other investigator's reports (Migliaccio et al., 1986, 1989), that tyrosine residues are phosphorylated in response to estrogen. In agreement with our results, other steroid hormone receptors are phosphorylated solely on serine residues in response to their cognate hormone (Housley and Pratt, 1983; Logeat et al., 1985; Pike and Sleator, 1985; Sullivan et al., 1988; Orti et al., 1989; Sheridan et al., 1989; Hoecd and Groner, 1990).

The rapid and ligand-specific manner of the phosphorylation of the estrogen receptor is similar to that observed for the progesterone receptor (Denner et al., 1990). However, multiple phosphorylation sites are observed on the progesterone and glucocorticoid receptors (Wei et al., 1987; Orti et al., 1989; Bodwell et al., 1991), whereas we found, by HPLC (Fig. 5B), one major and two minor [32P]phosphotryptic peptides of the estrogen receptor that are rapidly phosphorylated in response to estradiol. Under our experimental conditions the [32P]phosphate and [32P]ATP pools of the MCF-7 cells are at equilibrium; thus the estrogen-stimulated phosphorylation of the receptor is not a consequence of changes in an estrogen-stimulated [32P]ATP pool size. On the other hand, not all receptor phosphorylation sites are at equilibrium with the [32P]ATP pool but reflect estradiol-stimulated phosphorylation of specific sites. In addition, phosphorylation sites of the receptor that have a slower turnover or are constitutively phosphorylated would not be detected under these conditions. The two minor [32P]phosphopeptides detected by HPLC chromatography may be distinctly different phosphorylated (sites) peptides or more likely reflect heterogeneity of the major [32P] phosphopeptide eluting at 50 min, caused by peptide heterogeneity generated by the trypsin or by multiple phosphorylation sites on a single peptide. The simplicity of the estrogen receptor's phosphorylation pattern may facilitate assessing the biochemical consequences of its phosphorylation. It remains to be determined whether receptor phosphorylation is a prerequisite for DNA binding or occurs subsequently, and whether binding to specific DNA sequences is an additional requirement.

A relationship between the rapid estradiol-dependent phosphorylation of the receptor and its potential role in transcriptional activation is suggested by the analysis of the receptor's affinity for a specific DNA sequence (Fig. 6). The binding of the human or the calf estrogen receptor to a 32P-labeled DNA probe containing the consensus sequence of the estrogen-responsive element is greatly diminished by dephosphorylation of the receptor with potato acid phosphatase. Furthermore, upon addition of 10 mM phosphate, a competitive inhibitor of the phosphatase, the inhibition of receptor-ERE binding was attenuated. This strongly suggested that the phosphatase activity and not a contaminating proteolytic activity is responsible for the inhibition of receptor-ERE binding. Western blot analysis of the purified estrogen receptor following acid phosphatase treatment showed only the intact 67-kDa estrogen receptor which further demonstrates the absence of a proteolytic activity associated with the phosphatase preparation (data not shown).

The biochemical consequence of the receptor's phosphorylation and increased affinity for its ERE could greatly potentiate the action of the receptor by simply increasing the lifetime of the receptor-DNA complex, thus leading to more effective transcriptional activation. Recent findings have noted that phosphorylation of transcriptional factors may result in either an increase (Prywes et al., 1988) or decrease (Lamph et al., 1990; Kapiloff et al., 1991) in their affinity for specific DNA sequences. In addition, it has been noted that phosphorylation of transcriptional factors modulates transcriptional activity without effects on their affinity for the DNA (Taylor and Young, 1990; Tanaka and Herr, 1990). It also has been reported that binding of the SP-1 factor to its specific DNA sites is coupled with its phosphorylation and that this appears essential for transcriptional activation (Jackson et al., 1990). Importantly, the phosphorylation of the receptor may provide insight into the precise nature of the receptor-ERE interaction and the molecular processes that the receptor is involved in to initiate transcription.

Moreover, these findings indicate that phosphorylation pathways may exist that influence the interaction of the estrogen receptor with specific ERE by way of second messenger pathways or specific protein kinases. The determination and mutation of the exact serine residue(s) that are phosphorylated on the human estrogen receptor will provide insight into the mechanism of how phosphorylation regulates transcription.

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