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Phosphorylation of the Human Estrogen Receptor by Mitogen-activated Protein Kinase and Casein Kinase II: Consequence on DNA Binding

Steven F. Arnold,[1] John D. Obourn,[1]* Howard Jaffe[2] and Angelo C. Notides[1]†

[1]*Departments of Environmental Medicine and Biophysics, University of Rochester School of Medicine and Dentistry, Rochester, NY 1642 and* [2]*The LNC-NINDS Protein/Peptide Sequencing Facility, NIH, NINDS, Bethesda, MD 20892, U.S.A.*

We determined the amino acid and radiolabel sequences of tryptic [^{32}P]phosphopeptides of the purified human estrogen receptor (hER) from MCF-7 cells and Sf9 cells. Serine 118 was identified as a site that was phosphorylated independently of estradiol-binding in MCF-7 cells. Proline is on the carboxy terminus of serine 118, which suggests that the serine–proline may be a consensus phosphorylation site motif for either the mitogen-activated protein (MAP) kinase or p34^{cdc2} kinase. MAP kinase selectively phosphorylated the recombinant hER *in vitro* on serine 118 independent of estradiol-binding, whereas p34^{cdc2} did not phosphorylate the hER. We demonstrated previously that serine 167 of the hER was phosphorylated in an estradiol-dependent manner. We therefore compared the consequence of hER phosphorylation at serine 118 by MAP kinase and phosphorylation at serine 167 by casein kinase II on the receptor's affinity for specific DNA binding. The binding of the hER to an estrogen response element was not altered by phosphorylation with MAP kinase at serine 118 but was significantly increased when phosphorylated at serine 167 by casein kinase II. These data suggest that phosphorylation of the hER by MAP kinase(s) pathways may influence receptor action by a mechanism other than the estradiol-dependent phosphorylation of hER by casein kinase II.

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INTRODUCTION

The estrogen receptor is a member of a large superfamily of transcription factors that includes the steroid hormone and thyroid hormone, as well as the vitamin D and retinoic acid receptors. The members of this superfamily of nuclear receptors mediate changes in gene expression in response to hormone-binding [1]. The structural organization of these nuclear receptors include a small centrally located DNA-binding domain, a hormone-binding domain in the larger carboxyl terminus and a transcriptional activation domain in the amino terminus [2]. The estrogen receptor binds to specific DNA sequences referred to as estrogen responsive elements that are usually located in the 5′ region of responsive genes [3]. Steroid hormone receptors are regulated principally by hormone-binding. There is, however, evidence that phosphorylation may be another mechanism by which steroid hormone receptors are regulated [4]. Phosphorylation of the progesterone and thyroid receptors has been shown to increase DNA binding, while *in vitro* phosphorylation of NGFI-B by protein kinase A has been shown to repress DNA binding [5–7]. Phosphorylation has also been correlated with increased transcriptional activity of the vitamin D receptor and the thyroid receptor [6, 8]. Although, mutation analysis of the phosphorylation sites on the glucocorticoid receptor has not detected dramatic changes in the transcriptional activity of the receptor [9]. It has been suggested that phosphorylation of the glucocorticoid receptor influences

*Present Address: Haskell Laboratory for Toxicology and Industrial Medicine, Newark, DE 19714, U.S.A.
†Correspondence to A. C. Notides.
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the receptor's recycling between the cytoplasm and nucleus [10]. Therefore, while phosphorylation sites have been identified for the glucocorticoid, progesterone and vitamin D receptors, the physiological significance of phosphorylation of steroid hormone receptors remains unclear [8, 11, 12]. Findings that growth factors, activators of protein kinase A and C, and phosphatase inhibitors can induce hyperphosphorylation and increase the transcriptional activity of human estrogen and progesterone receptors complicate the picture further [13–15]. These results suggest that signaling pathways indirectly activate protein kinases, such as MAP kinase, that may in turn act upon steroid hormone receptors.

Phosphorylation of the estrogen receptor has been correlated with nuclear retention and specific DNA binding [16, 17]. Reports of mutational analyses have suggested that serines 104 and/or 106 and 118 of the hER are estrogen-dependent phosphorylation sites that modulate transcriptional activation [18, 19], while others have observed that phosphorylation of tyrosine(s) may be a prerequisite for hormone-binding [20]. Serine 167 has been identified as the major estrogen-dependent phosphorylation site on the hER overexpressed in Sf9 insect cells and in human mammary MCF-7 cells [21]. In addition, casein kinase II was shown to phosphorylate serine 167 on the hER in a hormone-dependent manner *in vitro* [21].

The present report describes the identification of serine 118 as a biologically relevant phosphorylation site on the recombinant and native hER. Furthermore, that MAP kinase phosphorylated the purified recombinant hER *in vitro* on serine 118. Binding of the hER to an estrogen response element was increased after phosphorylation by casein kinase II at serine 167 but not by phosphorylation with MAP kinase.

EXPERIMENTAL

Materials

The [^{32}P]orthophosphate (8500 Ci/mmol), [γ-^{32}P]ATP (3000 Ci/mmol) and 17β-[6,7^{3}H(N)]estradiol (45.6 mCi/mmol) were purchased from Dupont/New England Nuclear. Bovine calf serum (heat inactivated), okadaic acid, and bovine insulin were purchased from Sigma (St Louis, MO). The cyanogen bromide was from Pierce (Rockford, IL). The sequencing grade trypsin and the casein kinase II substrate peptide (RRREEETEEE) were from Promega (Madison, WI). The leupeptin, pepstatin, and chymostatin were obtained from Peninsula Laboratories (Belmont, CA). The MAP kinase (0.9 μmol PO_4 transferred/min/mg kinase) and p34^{cdc2} (0.6 μmol PO_4 transferred/min/mg kinase) were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). The casein kinase II (3 μmol PO_4 transferred/min/mg kinase) was a generous gift from Drs D. W. Litchfield and E. G. Krebs.

Purification of the hER from Sf9 or MCF-7 cells

The construction and expression of the recombinant baculovirus, AcNPV-hER carrying the cDNA of the hER has been described previously [22]. The purification of the hER was done as previously described with minor modifications [21]. All buffers used, except where noted without phosphatase inhibitors, contained 50 mM sodium fluoride, 10 mM sodium orthovanadate, 10 mM sodium pyrophosphate and 50 nM okadaic acid; in addition, the protease inhibitors 0.2 mM phenylmethylsulfonyl fluoride and 0.5 mM leupeptin were added. The buffers for the purification of the hER from MCF-7 cells also included 10 μg/ml of chymostatin and pepstatin. In addition, high speed supernatant from Sf9 cells infected with AcNPV-hER was added to the whole cell extract from MCF-7 cells in order to increase the hER concentration. The hER from ERE-Teflon chromatography was purified to homogeneity by separating the eluate by SDS–gel electrophoresis for 5 h at 30 mA. The ^{32}P-labeled hER was visualized by autoradiography and excised from the gel. The hER was electroeluted from the gel using the Elutrap (Schleicher & Schuell) with 15 mM ammonium carbonate and 0.1% SDS for 5 h at 200 V. The eluate from the electroelution was precipitated with 15% TCA for 16 h at 4°C. The TCA pellet was rinsed with acetone and allowed to dry.

[^{32}P]Orthophosphate labeling of the hER in Sf9 or MCF-7 cells

Sf9 cells in 150 cm^2 flasks were infected for 24–48 h with the AcNPV baculovirus and the viral media was replaced with phosphate-free minimal essential medium (Gibco, Grand Island, NY), pH 6.2, for 1 h. Each flask was incubated with 10 mCi of [^{32}P]orthophosphate for an additional 4 h and with 1 μM estradiol for 30 min. MCF-7 cells in 150 cm^2 flasks were incubated in phosphate-free minimal essential medium with 5% bovine calf serum containing 10 nCi [^{32}P]orthophosphate for 16 h at 37°C. The cells were then incubated with 50 nM estradiol or vehicle for 30 min. The hER from both sets of cells was purified as described above.

RP-HPLC analysis of the tryptic [^{32}P]phosphopeptides of the hER

The purified hER from Sf9 or MCF-7 cells was dissolved in 70% formic acid with CNBr (100 mg/ml) for 24 h in the dark at 21°C under argon. After digestion the sample was lyophilized and then washed with water. The CNBr digest was resuspended in 0.1% TFA with 6 M guanidine hydrochloride for RP-HPLC (Beckman System Gold) with a 0.21 × 25 cm C4 reverse phase column (Vydac, Hesparia, CA). The sample was washed for 20 min with 100% buffer A (100% water with 0.1% TFA) and then separated with a 1%/min gradient of buffer B (80% acetonitrile:20%

water with 0.08% TFA) at a flow rate of 500 μl/min. Fractions were collected every minute. the radioactivity was determined by Cerenkov counting and the peptide absorbance was at 220 nm. The ^{32}P-labeled CNBr peaks were combined, dried and washed with water. The peptides were reduced and alkylated according to the method of Stone *et al.* [23]; 1 μg of trypsin was added for every 25 μg of hER and allowed to digest for 24 h at 37°C. The digest was terminated by 10 μl of 100% TFA and separated by RP-HPLC with a 0.21 × 25 cm C18 reverse phase column (Vydac, Hesparia, CA). The sample was washed with 100% buffer A for 20 min and then separated with a gradient from 0% B to 37% B over 65 min, then from 37% B to 75% B for 20 min and 75% B to 100% B over 25 min at a flow rate of 500 μl/min. Fractions were collected at 1 min intervals and the radioactivity and peptide absorbance were determined as above.

Amino acid sequencing of the [^{32}P]phosphopeptides

The additional purification of the tryptic [^{32}P]phosphopeptides from the 2.1 × 250 mm C18 reverse phase column (Vydac, Hesperia, CA) was accomplished by rechromatography of the [^{32}P]phosphopeptides on a 1 × 100 mm C8 Aquapore RP-300 column (Applied Biosystems, Foster City, CA). Radioactive phosphopeptide fractions were stored at −70°C and allowed to decay to background. The decayed phosphopeptide fractions were applied in 30 μl aliquots to Biobrene (Applied Biosystems, Foster City, CA) treated glass fiber filters, dried, and subjected to automated Edman degradation on a Model 477A pulsed-liquid protein sequencer equipped with a Model 120A PTH analyzer (Applied Biosystems, Foster City, CA) using methods and cycles supplied by the manufacturer. Data was collected and analyzed on a Model 610A data analysis system (Applied Biosystems, Foster City, CA).

Radiolabel sequencing of the [^{32}P]phosphopeptides

The [^{32}P]phosphopetides were covalently bound to an aryl amine-derivatized PVDF disc using the parts and procedure supplied in the Millipore (Milford, MA) Sequelon AA attachment kit, # GEN920033. To remove unbound peptide after the coupling procedure was complete, the disc was washed alternately in 1 ml of water and 1 ml of methanol until background levels of radiation were detected in the washes. The disc was minced and placed in the reaction cartridge of the Model 477A pulsed-liquid protein sequencer (Applied Biosystems, Foster City, CA). A disc of Immobilon-P (Millipore, Milford, MA) was placed below the peptide. The radiosequencing was performed according to the methods and the cycles supplied by Applied Biosystems, with the exception that 90% methanol was used for S3.

In vitro protein kinase assays

Recombinant hER was purified as described [21] except that neither estradiol nor phosphatase inhibitors were added during purification, consequently the hER was dephosphorylated. The concentration of the purified recombinant hER was determined by [^{3}H]estradiol binding and Western blot analysis with the anti-hER antibody. Purified recombinant hER (10 pmol in 5 μl) was incubated on ice with 15 μl of p34^{cdc2} reaction buffer (80 mM HEPES, pH 7.4 and 10 mM $MgCl_2$) or 15 μl of MAP kinase reaction buffer (60 mM MOPS, pH 7.2, 50 mM β-glycerol phosphate, 2.0 mM EGTA, 30 mM $MgCl_2$, 0.2 mM NaF and 8 mM dithiothreitol) with 1.2 μM estradiol or vehicle for 1 h. Then, [γ-^{32}P]ATP (300 mCi/mmol) was added at a final concentration of 200 μM, 25 ng of p34^{cdc2} or 50 ng of MAP kinase were added to initiate the reaction in a final volume of 60 μl and incubated for 30 min at 30°C. The reaction was terminated by the addition of 10 μg of insulin and 10 μl of 100% TCA. The TCA precipitate was resuspended in Laemmli sample buffer [24] and subjected to SDS–gel electrophoresis for 5 h at 30 mA. The SDS–gel was dried and subjected to autoradiography at −70°C.

Gel mobility shift assay

Probe preparation and gel mobility shift assays were performed as previously described [25]. The recombinant hER was purified in the presence or absence of phosphatase inhibitors as described above. The hER purified in the absence of phosphatase inhibitors was phosphorylated as described above by MAP kinase or casein kinase II [21]. The hER (0.2 pmol) in 20 mM HEPES, pH 7.5, 1 mM EDTA, 150 mM KCl, 100 nM estradiol, 10% glycerol and protease inhibitors was incubated with the ^{32}P-labeled ERE and 500 ng of poly(dIdC) in 20 μl for 30 min at 4°C. Samples were loaded onto a pre-run 5% polyacrylamide gel followed by electrophoresis at 175 V for 3 h with cooling in 25 mM Tris buffer, pH 8.0, 152 mM glycine and 1 mM EDTA. The gel was dried and autoradiographed at −70°C.

RESULTS

Radiolabel and amino acid sequence of [^{32}P]phosphopeptides 5 and 6 from the recombinant hER

The ^{32}P-labeled recombinant and native hER, purified to homogeneity by ERE-affinity chromatography and SDS–gel electrophoresis, were digested with cyanogen bromide (CNBr) and trypsin. The [^{32}P]phosphopeptides were then separated by reverse phase-high performance chromatography (RP-HPLC). [^{32}P]Phosphopeptides 5 and 6, were common to both the recombinant and native hER, i.e. the corresponding phosphopeptide had the identical elution time and ^{32}P-labeling characteristics, were assumed to be the

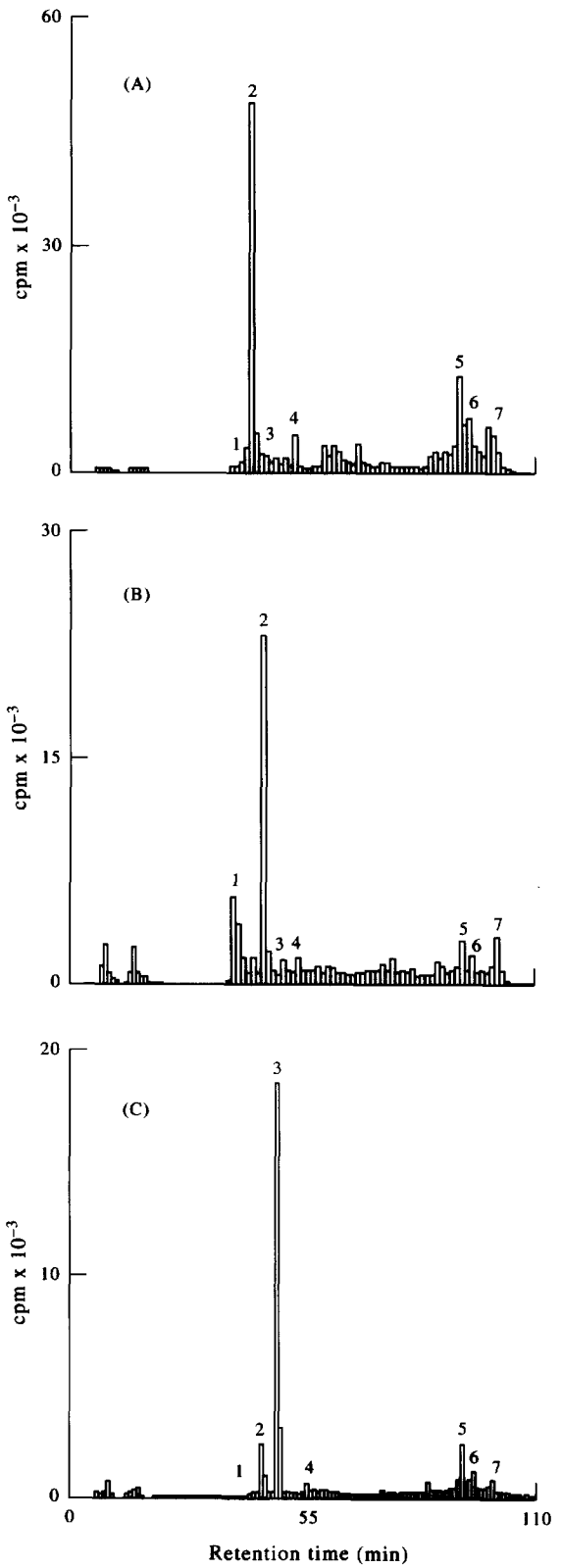

Fig. 1. RP-HPLC of the [^{32}P]phosphopeptides of the hER from Sf9 and MCF-7 cells. The ^{32}P-labeled hER from Sf9 cells and MCF-7 cells was purified, digested with CNBr and trypsin, then analyzed by RP-HPLC with a C18 column. (A) The recombinant hER from Sf9 cells treated with estradiol for 30 min. (B) The hER from MCF-7 cells treated with estradiol for 30 min. (C) The hER from MCF-7 cells not treated with estradiol.

identical phosphopeptide (Fig. 1). [^{32}P]Phosphopeptides 5 and 6 from the ^{32}P-labeled recombinant hER were isolated and sequenced. The majority of [^{32}P]phosphate from either [^{32}P]phosphopeptides 5 or 6 was released at the 9th residue during the Edman degradation [Fig. 2(A and B)]. This indicated that the 9th amino acid was phosphorylated on both [^{32}P]phosphopeptides 5 and 6. To identify [^{32}P]phosphopeptides 5 and 6, we further purified [^{32}P]phosphopeptides 5 and 6 by RP-HPLC with an Aquapore C8 column for amino acid sequencing. Amino acid sequencing of [^{32}P]phosphopeptide 5 and 6 revealed it to consist of approx. 100 pmol of a single peptide that included the amino acid residues 110–129 of the hER (Table 1). Taken together, the radiolabel and amino acid sequencing data lead us to conclude that both [^{32}P]phosphopeptides 5 and 6 contained serine 118 as a phosphorylation site.

[^{32}P]Phosphopeptides 5 and 6 were not sequenced in their entirety; nevertheless, it is reasonable to assume that [^{32}P]phosphopeptides 5 and 6 were generated because of differential digestion at the carboxyl terminus of the peptides. Both [^{32}P]phosphopeptides 5 and 6 start at leucine 110, resulting from a CNBr cleavage at methionine 109. In addition, we have detected digestion at tyrosine 130 when a peptide with amino acid 131–142 of the hER was sequenced (Fig. 3). The digestion of the tyrosine–tyrosine bond at 130–131 probably results from chymotryptic activity [26] present in the trypsin preparation. Therefore, if [^{32}P]phosphopeptides 5 and 6 are differentially digested at the carboxyl terminus digestion it most likely occurs at tyrosine 130 and arginine 142, respectively. Alternatively, [^{32}P]phosphopeptides 5 and 6 could have eluted differently from the RP-HPLC due to an additional site of phosphorylation besides serine 118. This is highly unlikely since the radiolabel sequence filter contained less than 2% of the starting [^{32}P]phosphate after sequence analysis suggesting that serine 118 is the only phosphorylated residue on [^{32}P]phosphopeptides 5 and 6.

Interestingly, [^{32}P]phosphopeptide 5 and 6 of the native hER from MCF-7 cells did not undergo an increase in phosphorylation after hormone binding [compare Fig. 1(B and C)]. [^{32}P]Phosphopeptides 5 and 6 contained 12% of the recovered [^{32}P]phosphate on the native hER from MCF-7 cells in the presence or absence of estradiol.

In vitro phosphorylation of the recombinant hER

Serine 118 is immediately followed by a proline, suggesting that this may be a consensus phosphorylation site motif for a cell cycle-dependent or a growth factor-stimulated protein kinase, such as p34^{cdc2} or MAP kinase [27]. We investigated this possibility by using recombinant hER purified in the absence of phosphatase inhibitors, (i.e. the dephosphorylated hER), which was 90% pure [22], as a substrate for

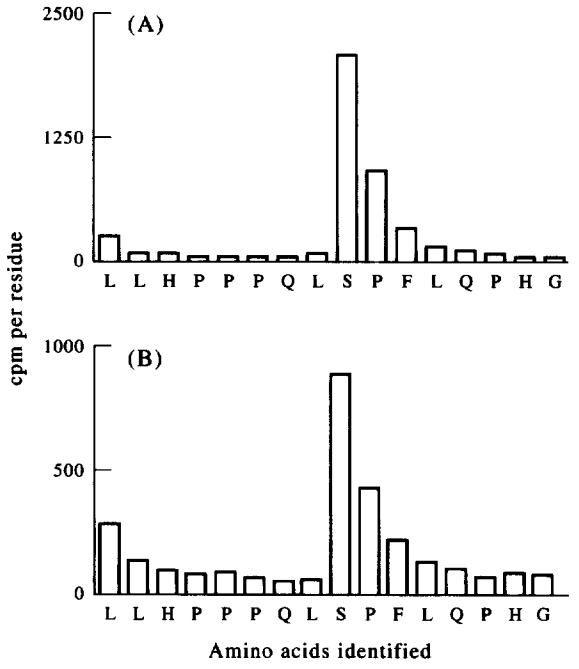

Fig. 2. Radiolabel and amino acid sequence of [^{32}P]phosphopeptides 5 and 6 from the recombinant hER. The ^{32}P-labeled recombinant hER was digested with CNBr and trypsin then analyzed by RP-HPLC with a C18 column. Radiolabel and amino acid sequencing was performed on the isolated [^{32}P]phosphopeptide 5 (A), and [^{32}P]phosphopeptide 6 (B). The amino acid sequences identified by Edman amino acid sequencing are shown and correspond to hER residues 110–129.

p34^{cdc2} and MAP kinase. The ^{32}P-labeled recombinant hER purified in the absence of phosphatase inhibitors was completely devoid of [^{32}P]phosphate indicating the hER was dephosphorylated during purification (data not shown). The purified recombinant hER was incubated with [γ-^{32}P]ATP and either p34^{cdc2} or MAP kinase, then precipitated with TCA followed by SDS–gel electrophoresis and transfer to a PVDF membrane. The autoradiography revealed that the p34^{cdc2} did not phosphorylate the recombinant hER, but incorporated [^{32}P]phosphate into other protein bands indicating that the protein kinase was active (Fig. 4). MAP kinase and casein kinase II, are contaminants in the purified hER preparation (detected by Western blot analysis) which were phosphorylated by the p34^{cdc2} (Fig. 5, data not shown). The contaminant MAP kinase from Sf9 cell extract was not active in the hER sample prepared in the absence of phosphatase inhibitors since the MAP kinase was dephosphorylated and requires phosphorylation on threonine and tyrosine residues for activity [28].

In contrast, the addition of purified active MAP kinase to the recombinant hER did phosphorylate the hER (Fig. 5). The [^{32}P]phosphate incorporated into the purified hER by MAP kinase was maximal at 50 ng of the MAP kinase and incorporated 0.7 mol of [^{32}P]phosphate/mol hER.

Next, we identified which site(s) on the recombinant hER were phosphorylated *in vitro* by MAP kinase. The purified recombinant hER was [^{32}P]phosphorylated with MAP kinase and resolved by SDS–gel electrophoresis. The ^{32}P-labeled hER was digested with CNBr followed by trypsin. The RP-HPLC analysis of the [^{32}P]phosphopeptides of the recombinant hER reproducibly revealed two [^{32}P]phosphopeptides whose retention time was identical to that of [^{32}P]phosphopeptides 5 and 6, i.e. serine 118 (Fig. 6). The *in vitro* phosphorylation of the recombinant hER by MAP kinase was highly specific. More than 85% of the [^{32}P]phosphate incorporated into the hER was recovered in phosphopeptides 5 and 6. Since the phosphorylation of the purified recombinant hER by the MAP kinase was specific for serine 118, we next examined whether MAP kinase preferred to phosphorylate the liganded, as opposed to the unliganded, hER. The MAP kinase phosphorylated the liganded and the unliganded hER equally well as determined by Cerenkov counting of the excised hER bands (Fig. 5, lanes 2 and 3).

Gel mobility shift assay of the hER

We previously reported that dephosphorylation of the hER with potato acid phosphatase reduced its

Table 1. Automated Edman analysis of phosphopeptide 6

Amino acid identified	Yield (pmol)*	Corresponding residue number
Leu	91.52	110
Leu	71.00	111
His	10.12	112
Pro	61.64	113
Pro	69.40	114
Pro	60.64	115
Gln	32.63	116
Leu	45.19	117
Ser	12.17†	118
Pro	24.71	119
Phe	35.14	120
Leu	33.66	121
Gln	10.29	122
Pro	12.13	123
His	1.94	124
Gly	5.29	125
Gln	6.97	126
Gln	8.49	127
Val	6.99	128
Pro	4.95	129

*Lag corrected yields.

†The presence of PTH-serine at the 9th residue results from the coelution of the phosphorylated and non-phosphorylated peptide 110–129. Nevertheless, the presence of phosphoserine is also suggested by elevated levels of PTH-dehydroalanine in the 9th cycle (not shown), resulting from the β-elimination reaction of phosphoserine. The sequence of phosphopeptide 5 was identical (data not shown).

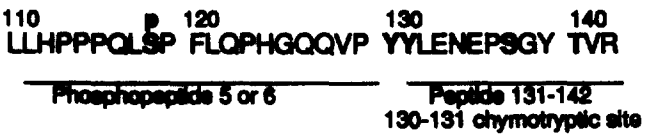

Fig. 3. Alignment of hER sequence of phosphopeptide 5 and 6 with peptide 131–142. The amino acid sequence analysis of approx. 100 pmol of [^{32}P]phosphopeptides 5 and 6 both identified residues 110–129 of the hER. The radiolabel sequencing of [^{32}P]phosphopeptides 5 and 6 identified the ninth residue, serine 118 as the phosphorylated residue. Approximately 40 pmol of peptide 131–142 was found. Peptide 131–142 was not radiolabeled, indicating that serine 137 and threonine 140 were not phosphorylated. Chymotryptic activity hydrolyzed the tyrosine–tyrosine bond at 130–131.

affinity for an estrogen response element (ERE) in a gel mobility shift assay [17]. The recombinant hER purified in the presence or absence of phosphatase inhibitors (i.e. phosphorylated hER or dephosphorylated hER, respectively) revealed that the phosphorylated hER bound 10-fold more ^{32}P-labeled ERE than the dephosphorylated hER (Fig. 7). The addition of whole cell extract from uninfected Sf9 cells did not enhance the binding of the dephosphorylated purified hER to the ERE. The dephosphorylated hER was phosphorylated by MAP kinase or casein kinase II and then assayed for DNA binding. The binding of the hER to an ERE after phosphorylation by MAP kinase did not change. In contrast, the binding of the hER to an ERE after phosphorylation by casein kinase II increased in a dose-dependent manner. Maximal binding of hER to the ERE was achieved after phosphorylation of the hER with 25 ng of casein kinase II and was approximately equivalent to the binding observed for the hER purified in the presence of phosphatase inhibitors. Casein kinase II alone, in the absence of ATP, did not increase the binding of the hER to the ERE; suggesting the binding to the ERE was dependant upon serine 167 phosphorylation (data not shown). The increase in DNA binding of the hER by casein kinase II was inhibited by a casein kinase substrate peptide, further demonstrating that the phosphorylation of the

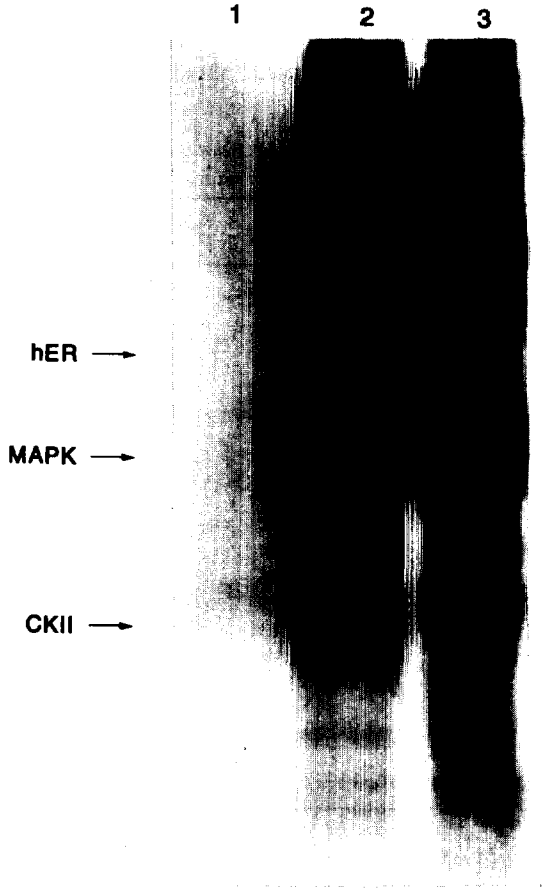

Fig. 4. *In vitro* incubation of the purified recombinant hER with p34^{cdc2}. Purified recombinant hER (10 pmol) was incubated with p34^{cdc2} and [γ-^{32}P]ATP, the products were then resolved by SDS–gel electrophoresis and visualized by autoradiography. The purified recombinant hER was treated with estradiol but without p34^{cdc2} (lane 1); without estradiol and with p34^{cdc2} (lane 2); or with estradiol and p34^{cdc2} (lane 3). The casein kinase II (CKII) was detected by Western blot analysis.

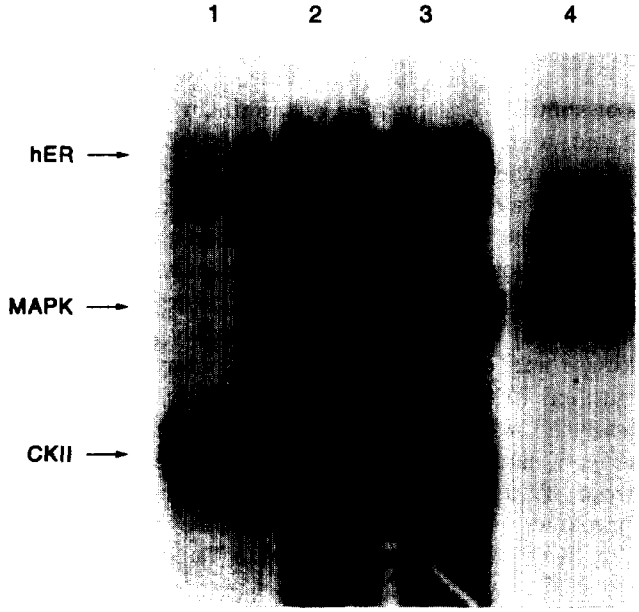

Fig. 5. *In vitro* phosphorylation of the purified recombinant hER by MAP kinase. Purified recombinant hER (10 pmol) was incubated with MAP kinase and [γ-^{32}P]ATP and the products were resolved by SDS–gel electrophoresis and visualized by autoradiography. The purified recombinant hER was treated: with estradiol but without MAP kinase (lane 1); without estradiol and with MAP kinase (50 ng) (lane 2); with estradiol and MAP kinase (lane 3); and MAP kinase alone (lane 4).

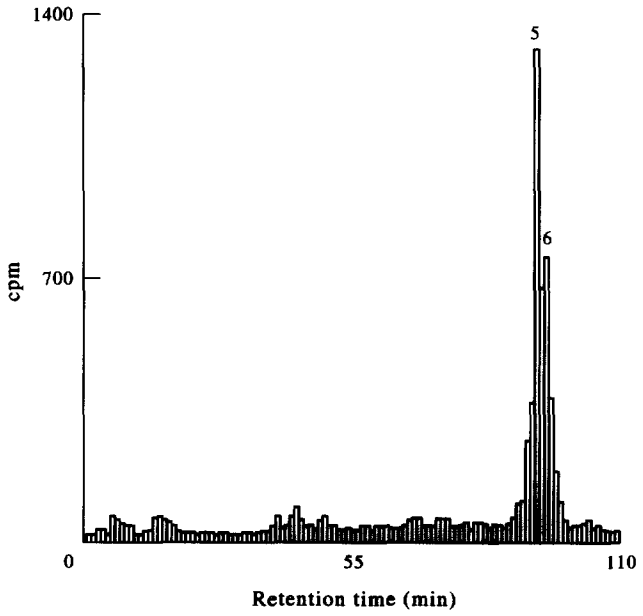

Fig. 6. RP-HPLC analysis of the [^{32}P]phosphopeptides from the recombinant hER phosphorylated *in vitro* by MAP kinase. Purified recombinant hER (10 pmol) was phosphorylated by MAP kinase (50 ng) and resolved by SDS–gel electrophoresis. The ^{32}P-labeled recombinant hER from 10 separate lanes of the SDS gel were eluted, pooled, digested with CNBr and trypsin, and analyzed by RP-HPLC.

hER by casein kinase II was responsible for the increase in DNA binding (Fig. 7).

DISCUSSION

We have shown by radiolabel and amino acid sequence analyses that the hER is phosphorylated on serine 118 and that the phosphorylation is not enhanced by estradiol-binding. Consistent with the *in vivo* findings, our *in vitro* MAP kinase studies show that specific phosphorylation of the hER at serine 118 is independent of estradiol-binding. Interestingly, the sequence on the hER phosphorylated by MAP kinase, gln-leu-ser-pro, is identical to the MAP kinase phosphorylation site of the nuclear protein, TAL1 [29]. MAP kinase also phosphorylated the recombinant human glucocorticoid receptors (hGR) and progesterone receptor (hPR) *in vitro*, as might be expected since the majority of the phosphorylation sites identified for the hPR and hGR are serine–proline motifs [11, 12]. Interestingly, MAP kinase incorporated approx. 1 mol of phosphate into the hPR whereas, 2 mol of phosphate were incorporated into a mol of the hGR receptor (data not shown). The cell cycle specific protein kinase, p34^{cdc2} did not phosphorylate the hER *in vitro*, not surprisingly since serine 118 does not contain a basic residue on its carboxyl terminus as is found for the consensus phosphorylation site motif of p34^{cdc2} [27].

The phosphorylation of both serines 118 and 167, in the amino-terminus of the hER are consistent with the amino-terminus localization of the phosphorylation sites on the glucocorticoid and progesterone receptors [11, 12, 21]. These findings support the idea that the regulation by phosphorylation of the steroid hormone receptors is accomplished by a small select group of protein kinases. These may include MAP kinase, casein kinase II and double-stranded DNA protein kinase which phosphorylate multiple members of the steroid hormone receptors, e.g. hER, hGR, hPR, human vitamin D receptor and the chicken erb-A receptor [21, 30–32]. Protein kinases A and C, however, do not phosphorylate the hER [33].

Ali *et al.* [19], using deletion and point mutants of the hER-cDNA that were transiently transfected into COS-1 cells, reported that serine 118 was phosphorylated in response to ligand-binding and that mutation of serine 118 to alanine decreased the transcriptional activity of the hER by 30–40%. Le Goff *et al.* [18], using the same strategy with COS-1 cells concluded that serines 104 and/or 106, in addition to serine 118, were phosphorylated and involved in transcriptional activation of the receptor. We have not detected phosphorylation of serine 104 or 106 on the hER expressed in either Sf9 or MCF-7 cells (data not shown). There are several plausible explanations for the differences between our findings and the reports of others [18, 19]. We have used amino acid and radiolabel sequencing of the hER to directly identify the phosphorylation sites. Our results may be hampered by the instability of the [^{32}P]phosphate on serine 118 during the purification of the hER. This seems unlikely, since we have included the phosphatase inhibitors sodium phosphate, fluoride, vanadate and okadaic acid in the buffers. Alternatively, the use of deletion and point mutants of the hER by Ali *et al.* [19] and Le Goff *et al.* [18] may have altered the structure of the receptor and subsequently its phosphorylation. For example, the major estradiol phosphorylation site on the recombinant and native hER is serine 167 [21] which was not identified as a phosphorylation site on the hER transfected into COS-1 cells [18, 19]. The MCF-7 cells are an estrogen responsive breast tumor cell line which constitutionally express the hER. Thus, the MCF-7 cells serve as a control to assess possibly spurious phosphorylation of the hER in other transfected cells such as, Sf9 or COS-1 cells. On the other hand, the differential phosphorylation patterns of the hER observed in different cell lines may not result from spurious phosphorylations but may be the basis of the cell and promoter specific activation of transcription by the receptor [34].

We believe our finding that the hER is phosphorylated by MAP kinase are consistent with reports that steroid hormone receptors are transcriptionally activated following mitogenic growth factor exposure. Several groups have reported [13–15] that steroid hormone receptors display increased phosphorylation and transcriptional activity after treating cells or tissues with epidermal growth factor or insulin-like growth factors I and II. A large body of evidence suggests that

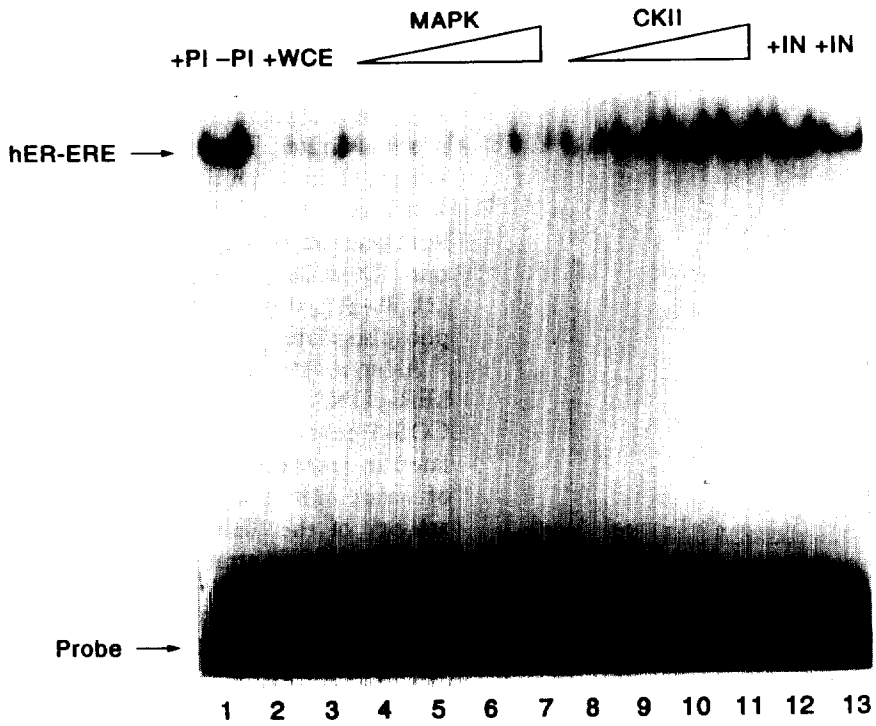

Fig. 7. A gel mobility shift assay of hER binding to an ERE following phosphorylation of the hER with casein kinase II or MAP kinase. Purified recombinant hER (0.2 pmol) was incubated with 25 fmoles of a 47 bp ^{32}P-labeled ERE from the chicken vitellogen II gene in 20 μl at 4°C for 30 min. Lane 1 contains hER purified in the presence of phosphatase inhibitors (+PI), lane 2 is the hER purified in the absence of phosphatase inhibitors (−PI). Lane 3 is the hER purified in the absence of phosphatase inhibitors plus whole cell extract (WCE). Lanes 4–7 are the hER purified in the absence of phosphatase inhibitors and phosphorylated with 10, 20, 30, 50 ng of MAP kinase, respectively. Lanes 8–11 are the hER purified in the absence of phosphatase inhibitors and phosphorylated with 5, 10, 15, 25 ng of casein kinase II, respectively. Lanes 12 and 13 are the hER phosphorylated by casein kinase II (25 ng) in the presence of 10 and 50 ng of a casein kinase II substrate peptide, respectively.

these growth factors act on signaling pathways that function as autocrine and paracrine mechanisms, which may also be important in cell transformation and tumor growth [35]. In numerous cell lines, growth factors, such as epidermal growth factor, have been shown to activate MAP kinase by phosphorylation [28]. We (data not shown) and others [36] have shown the existence of MAP kinase in Sf9 cells indicating that it is highly conserved throughout evolution and may phosphorylate serine 118 *in vivo*.

Our results and those of others suggest the following model for the activation of steroid hormone receptors following treatment with mitogenic growth factors, activators of protein kinase C and A and phosphatase inhibitors. The phosphorylation of steroid hormone receptors, resulting from ligand-independent pathways such as by MAP kinase, may impart qualitatively different transcriptional capabilities to steroid hormone receptors that after hormone-binding. Therefore, the steroid hormone receptors may regulate gene expression not only on the basis of hormone-binding, but also in the context of its cellular milieu, e.g. activated MAP kinase or AP-1 [37]. Evidence has shown that activators of protein kinase C and protein kinase A can promote the phosphorylation and transcriptional activation of the estrogen, progesterone and glucocorticoid receptors [14, 15]. Recently, it has been shown that protein kinase C and protein kinase A can activate upstream regulators (e.g. raf-1) of MAP kinase [38, 39].

To date, much of the effort to understand the role of phosphorylation on the functioning of the steroid hormone receptors has utilized mutagenesis studies [9, 18, 19]. We have used purified hER to identify protein kinases that phosphorylate physiological relevant sites. The observation that phosphorylation of serine 167 on the hER by casein kinase II increased the binding of the receptor to an ERE is novel. The increase of *in vitro* DNA binding by the hER after its phosphorylation by casein kinase II is consistent with the increase in DNA binding and receptor phosphorylation observed after MCF-7 cells were treated with estradiol (unpublished observations). The finding that phosphorylation of the hER by MAP kinase does not affect DNA binding suggests that the phosphorylation on serine 118 may serve to regulate other activities of the receptor, such as transactivation. Hormone-binding by the hER may change the conformation or the charge of the domain that includes phosphoserine 118,

resulting in the formation of an acidic transactivating domain [40].

In conclusion, we have identified serine 118 of the hER as a biological relevant phosphorylation site that is not regulated by estradiol. The DNA binding of the hER was found to increase with phosphorylation of serine 167 by casein kinase II. The demonstration that phosphorylation of the hER *in vitro* by MAP kinase suggests a mechanism by which polypeptide hormones may modulate steroid hormone action.

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