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In Vivo and *In Vitro* Phosphorylation of the Human Estrogen Receptor

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We report here that the human estrogen receptor (hER) overexpressed in Sf9 insect cells is phosphorylated similarly to hER from the human MCF-7 mammary carcinoma cell line. The recombinant and native hER labeled to steady-state with [^{32}P]phosphate were purified to homogeneity using specific DNA-affinity chromatography followed by SDS–gel electrophoresis. Resolution of the hER tryptic digests by reverse phase-high performance liquid chromatography revealed that five [^{32}P]phosphopeptides from the hER expressed in the Sf9 cells had retention times identical to five of the seven [^{32}P]phosphopeptides from the hER in MCF-7 cells. Uniquely, a dephosphorylation of a single ^{32}P-labeled peptide occurred in response to estradiol treatment of MCF-7 cells. *In vitro* protein kinase assays with the purified recombinant hER revealed that the DNA-dependent protein kinase (DNA-PK) phosphorylated the receptor and induced a decrease in the receptor's mobility as demonstrated by SDS–gel electrophoresis. In contrast, protein kinases A and C did not phosphorylate the purified recombinant hER. These results suggest that in the process of becoming transcriptionally active the estrogen receptor undergoes a dephosphorylation after estrogen-binding and subsequent phosphorylations, in part by the DNA-PK.

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INTRODUCTION

The human estrogen receptor (hER) is a member of the steroid/thyroid hormone receptor superfamily of ligand-activated transcription factors. All the members of this family share a common set of domains with each domain having a conserved function [1]. The amino terminal contains a hormone-independent, yet promoter- and cell-specific, transactivation function [2]. The carboxyl terminus contains the hormone binding domain, a dimerization motif and a hormone dependent transactivation function [2–4]. The centrally located DNA binding domain confers sequence specific DNA binding of the hER to its estrogen response element, ERE [3]. It has been previously shown that hormone binding, both *in vivo* and *in vitro* enhances the affinity of steroid hormone receptors for their respective response elements which usually are located in the 5′ region of steroid hormone responsive genes [5–7]. It has become increasingly clear that, aside from hormone binding, receptor phosphorylation is another mechanism that may contribute to the activity of steroid hormone receptors *in vivo* [8]. Phosphorylation of transcription factors has been shown to control a variety of functions such as, protein–protein interactions, DNA binding and transcriptional activation [9–12]. Steroid hormone receptors exist as phosphoproteins in the absence of hormone and undergo a hyperphosphorylation after hormone treatment which correlates with their biological activity *in vivo* [8, 13]. Growth factors, activators of protein kinase C and A and phosphatase inhibitors also result in the hyperphosphorylation of steroid hormone receptors [14–16]. Recent evidence suggests that DNA binding, transcriptional activation, ligand binding and even recycling [17–20] of steroid hormone receptors may be regulated by post-translational phosphorylation.

Similar to other steroid hormone receptors, estradiol treatment induces an increase in [^{32}P]phosphate incorporation onto serine residues of the estrogen receptor

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from calf uterus and the hER from MCF-7 cells [21]. The hyperphosphorylation of the hER has been correlated with increased nuclear retention and DNA binding, suggesting that the active form of the estrogen receptor in the nucleus is phosphorylated [21, 22]. Using mutational analysis of the hER, serine 118 has been identified as a potential hormone-dependent phosphorylation site that modulates transcriptional activation, while tyrosine 537 has been suggested to regulate hormone binding [19, 23, 24]. However, direct phosphopeptide sequence analysis of the hER will be necessary both to confirm these findings and to identify other possible phosphorylation sites.

To identify the phosphorylation sites we have used the baculovirus expressed hER in Sf9 insect cells [25]. Using this expression system and human MCF-7 cells, serine 167 was identified as the major estradiol-induced phosphorylation site of the hER [26]. We report here that following the long-term [^{32}P]orthophosphate labeling of the native hER from MCF-7 cells a previously unrecognized dephosphorylation of a specific site(s) on the hER occurs in response to hormone-binding. In addition, the purified recombinant hER was shown to be a target for the DNA-dependent protein kinase (DNA-PK) *in vitro*.

EXPERIMENTAL

Materials

The [^{32}P]orthophosphate (8500 Ci/mmol) and 17β-[6,7^{3}H(N)] estradiol (45.6 mCi/mmol) were purchased from Dupont/New England Nuclear. Fetal bovine and bovine calf serum (heat inactivated), okadaic acid, and Salmon sperm DNA were purchased from Sigma (St Louis, MO, U.S.A.). The cyanogen bromide was from Pierce (Rockford, IL). The sequencing grade trypsin was from Promega (Madison, WI). The leupeptin, pepstatin, and chymostatin were obtained from Peninsula Laboratories (Belmont, CA). The cAMP-dependent protein kinase catalytic subunit (1.2 μmol PO_4/min/mg) and protein kinase C (1 μmol PO_4/min/mg) were purchased from Upstate Biotechnology (Lake Placid, NY).

Antibody 6 purification and immunoprecipitation of the recombinant hER

The production of antibody 6 raised against a peptide corresponding to amino acid residues 259–278 of the hER has been described previously [25]. Antibody 6 was purified by attaching the hER peptide which included a N-terminal cysteine residue to a SulfoLink coupling gel (Pierce, Rockford, IL). The rabbit antiserum was incubated with the SulfoLink-peptide column at 21°C for 1 h. The column was washed with 20 vol of PBS, pH 7.4. The antibody was eluted with 100 mM glycine, pH 2.8 and collected into 3 M Tris, pH 8, to neutralize the pH of the eluate. The antibody was precipitated with 40% ammonium sulfate and centrifuged, then dissolved and dialyzed against 0.1 M phosphate, pH 7.4, for 16 h at 4°C. The final concentration of the antibody was determined by absorbance at 280 nm.

The medium from Sf9 cells infected with the AcNPV–hER baculovirus for 1–2 days was removed and replaced with serum-free phosphate-free minimal essential medium, pH 6.2 for 1 h. Five mCi of [^{32}P]orthophosphate was added and allowed to incubate for 4 h, then estradiol was added for 30 min. A high speed cytosol was prepared and incubated with purified antibody 6 for 16 h at 4°C. Protein-A-Sepharose (Pharmacia, Piscataway, NJ) equilibrated in Hank's Balanced Salt Solution (Gibco, Grand Island, NY) containing 10% non-fat milk was added for 2 h at 4°C. The Protein-A-Sepharose–antibody complex was centrifuged, washed twice with homogenization buffer containing 75 mM KCl and twice with homogenization buffer containing 300 mM KCl. The antibody complex was denatured with Laemmli loading buffer and the proteins resolved by SDS–gel electrophoresis with a 10% gel at 30 mA per gel for 5 h [27]. The proteins in the gel were electrotransferred to an Immobilon-P or polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA) using 3-[cyclohexylamino]-1-propanesulfonic acid (Sigma, St Louis, MO), pH 11, at 50 mA for 16 h. The autoradiograph was analyzed by scanning laser densitometry. The hER was also excised from the membrane and the radioactivity measured by Cerenkov counting. The amount of hER on the membrane was determined by Western blot analysis.

Western blot analysis of the hER

Whole cell extracts from MCF-7 or Sf9 cells containing 50 fmol of hER were separated by SDS–gel electrophoresis in a 10% acrylamide gel at 30 mA per gel for 10 h and then electrotransferred to an Immobilon-P membrane. The membrane was probed with a 1:2000 dilution of antibody 6 and a secondary peroxidase conjugated anti-rabbit IgG (Vector Laboratories, Burlingame, CA), the bands were visualized by chemiluminesence using the ECL system (Amersham, Arlington Heights, IL). The ER was quantified by scanning laser densitometry.

Labeling of the hER with [^{32}P]orthophosphate and analysis of the [^{32}P]phosphopeptides

The Sf9 cells were infected for 24–48 h with the recombinant AcNPV–hER baculovirus containing the cDNA of the hER, then the media was removed and replaced with phosphate-free minimal essential medium, pH 6.2, for 1 h. Each flask was then incubated with 10 mCi of [^{32}P]orthophosphate for 4 h and with 1 μM estradiol containing 10 nM [^{3}H]estradiol for 30 min. Confluent MCF-7 cells (150 cm^2 flasks) were incubated in phosphate-free minimal essential medium with 5% bovine calf serum containing 10 mCi of [^{32}P]orthophosphate per flask for 16 h at 37°C. The

cells were then incubated with 50 nM estradiol plus 5 nM [^{3}H]estradiol or vehicle alone for 30 min. The cells were then washed, lysed and the hER purified by estrogen response element (ERE)-affinity chromatography as previously described [25, 26]. Briefly, the cytosol of the Sf9 or the MCF-7 cells was prepared in and purified in a 40 mM Tris buffer, pH 7.4, containing phosphatase inhibitors (50 mM sodium fluoride, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate and 50 nM okadaic acid) and protease inhibitors (0.2 mM phenylmethanesulfonyl fluoride, 0.5 mM leupeptin, 1 mg/ml of pepstatin and chymostatin). The cytosol was made 40% saturated with respect to ammonium sulfate and the precipitate collected by centrifugation. The ammonium sulfate precipitate was dissolved in 40 mM Tris (pH 7.4), 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 100 mM KCl and the phosphatase and protease inhibitors listed above. Then 50 mg/ml of single- and double-stranded DNA was added to block nonspecific protein binding to the ERE-matrix. The hER was then bound to the ERE-containing oligonucleotide Teflon matrix for 1 h at 4°C. The ERE-matrix was washed with 30 vol of the Tris buffer containing 0.2 M KCl and eluted with the Tris buffer containing 0.6 M KCl and 10% glycerol. The hER was purified to homogeneity by SDS–gel electrophoresis [25, 26].

The hER was digested with cyanogen bromide and the [^{32}P]phosphopeptides were isolated by reverse phase-high performance liquid chromatography (RP-HPLC) using a C4 column. The cyanogen bromide [^{32}P]phosphopeptides were then redigested with trypsin and resolved by RP-HPLC with a C18 column [26].

In vitro phosphorylation of the recombinant hER by the DNA-PK, protein kinase A and protein kinase C

The recombinant hER was purified by ERE-affinity chromatography in the absence of phosphatase inhibitors [25, 26]. The purified hER (50 fmol) was incubated with 4 μl of purified DNA-PK protein kinase, 50 ng of a 26 bp ERE from the chicken vitellogenin II gene or 50 ng of sonicated salmon sperm DNA (Sigma, St Louis, MO) and 0.3 mM [γ-^{32}P]ATP (30 mCi/mmol) as described by Carter *et al.* [28]. The protein kinase A assay was in a buffer containing 20 mM Tris (pH 7.4), 1 mM EGTA, 5 mM $MgCl_2$, 0.3 mM [γ-^{32}P]ATP (30 mCi/mmol) with 100 ng of the cAMP-dependent catalytic subunit. The protein kinase C assay was in 20 mM Tris (pH 7.4), 0.1 mM $CaCl_2$, 5 mM $MgCl_2$, 0.31 mg/ml L-α-phosphatidyl-L-serine, 60 μg/ml 1,2-diolein, 0.03% Triton X-100, 0.3 mM [γ-^{32}P]ATP (30 mCi/mmol) with 100 ng protein kinase C. All reactions were terminated by TCA precipitation and the products resuspended in Laemmli sample buffer and analyzed by SDS–gel electrophoresis at 30 mA for 5 h [27]. The SDS–gel was transferred to an Immobolin-P membrane for autoradiography and Western blot analysis.

RESULTS

The recombinant hER is a phosphoprotein

The recombinant hER from Sf9 insect cells incubated with [^{32}P]orthophosphate revealed that the hER was phosphorylated. The recombinant hER was purified from the Sf9 cell extract by immunoprecipitation with an anti-hER antibody, followed by SDS–gel electrophoresis, and then transferred to a PVDF membrane. Autoradiography of the membrane revealed that the hER was a phosphoprotein as indicated by [^{32}P]phosphate incorporation while the Western

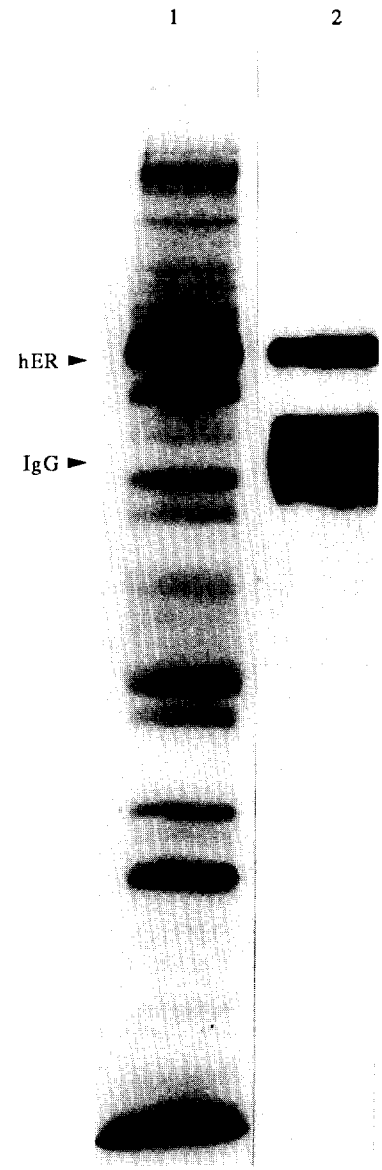

Fig. 1. Phosphorylation of the hER expressed in Sf9 cells. The Sf9 cells were infected with the baculovirus AcNPV–hER for 24 h, labeled with [^{32}P]orthophosphate for 4 h and treated with 1 μM estradiol for 30 min. The ^{32}P-labeled hER was immunoprecipitated with antibody 6, resolved by SDS–gel electrophoresis and then transferred to a PVDF membrane. The autoradiograph (lane 1) and Western blot (lane 2) of the immunopurified recombinant hER are shown. The IgG refers to the antibody used to immunoprecipitate the hER.

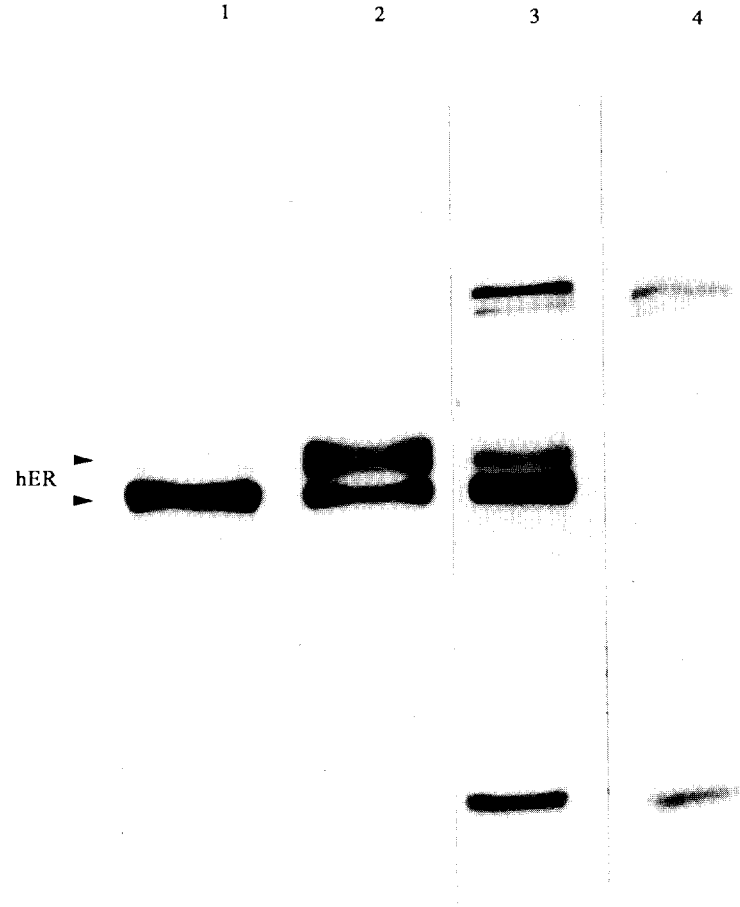

Fig. 2. The estradiol-induced SDS–gel electrophoretic upshift of the recombinant hER and native hER. Sf9 and MCF-7 cell extracts were subjected to SDS–gel electrophoresis and then electrotransferred to a PVDF membrane. The membrane was blotted with antibody 6 and visualized by a chemiluminesence method. The MCF-7 cells were treated with ethanol vehicle (lane 1) or 50 nM estradiol for 30 min (lane 2). The Sf9 cells at 24 h following baculoviral infection were treated with 1 μM estradiol for 30 min (lane 3). Mock infected Sf9 whole cell extract is shown (lane 4). Each lane contained 50 fmol of hER and 150 μg of protein.

blot analysis confirmed the identity of the hER (Fig. 1). In addition, the phosphoamino acid analysis of the recombinant hER revealed that serine was the only amino acid phosphorylated (data not shown). This result is in agreement with previous evidence that showed the estrogen receptors from calf uterus, MCF-7 cells [21] and the mouse uterus [22] are phosphoproteins. This is also consistent with previous observations that the progesterone [29] and glucocorticoid receptors [30] were phosphorylated in Sf9 cells.

A phosphorylation dependent decrease in the electrophoretic mobility of both the native hER and the recombinant hER was detected by Western blotting (Fig. 2). The hER from MCF-7 cells in the absence of estradiol exists predominately as a single band migrating at 66 kDa. Treatment of the MCF-7 cells with 50 nM estradiol for 30 min resulted in an upshift of approx. 60% of the receptor band to a slower migrating form at 67 kDa. The 67 kDa form represents the hyperphosphorylated form of the hER since phosphatase treatment caused selective collapse of this form of the hER to the 66 kDa species (C. Hurd and A. Notides, unpublished results).

The recombinant hER from Sf9 cells treated with estradiol also showed two electrophoretic forms. The recombinant hER from Sf9 cells exists as approx. 30% in the 67 kDa form and 70% in the 66 kDa form (Fig. 2). The 67 kDa form of the recombinant hER also collapsed into the 66 kDa form following phosphatase treatment (data not shown). Thus, the hER from MCF-7 and Sf9 cells exist as phosphoproteins as shown by [^{32}P]phosphate incorporation and decreased electrophoretic mobility.

These data suggest that the recombinant hER produced in Sf9 cells is a useful system for identifying the phosphorylation sites on the hER. Production of the recombinant hER in milligram quantities [25] is ideally suited for phosphopeptide mapping, radiolabel and amino acid sequencing of phosphopeptides. From approx. 1 mg of recombinant receptor 100–200 μg of

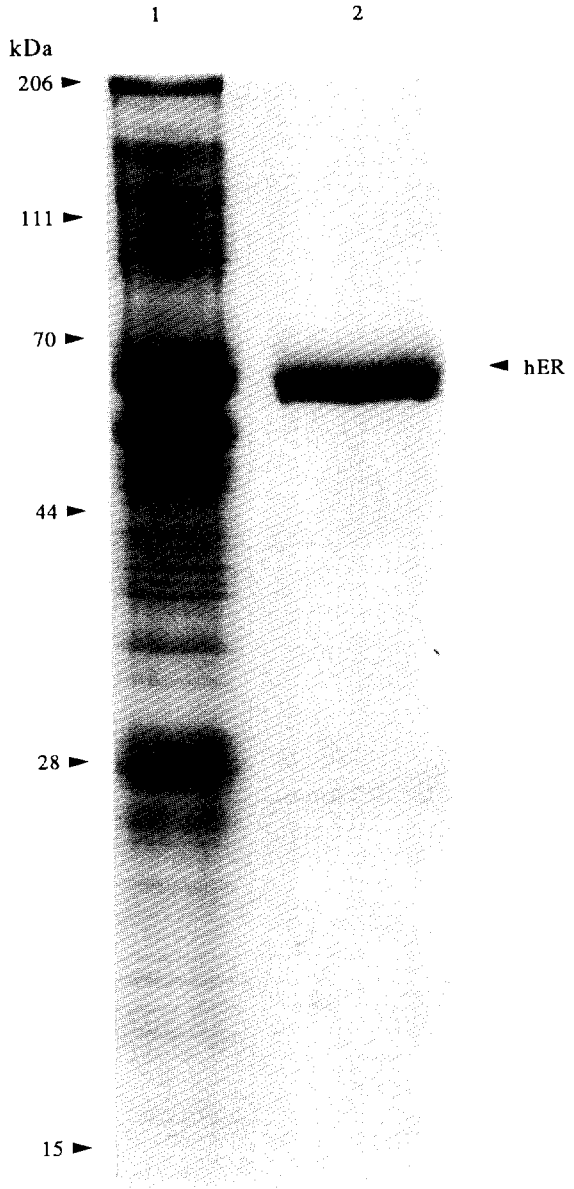

Fig. 3. Purification of the recombinant hER with a ERE–Teflon column. The 40% ammonium sulfate fraction of the Sf9 cell extract (lane 1) and the purified recombinant hER (50 μg) eluted from the ERE–Teflon column (lane 2) are shown following SDS–gel electrophoresis. The gel was stained with Coomasie Blue.

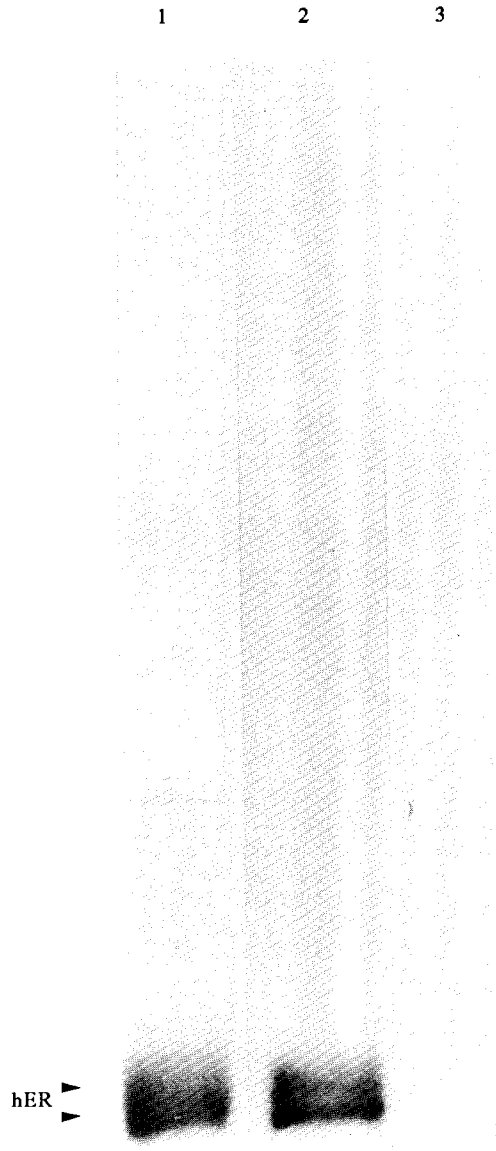

Fig. 4. The recombinant hER before and after ERE–Teflon purification. The recombinant hER was precipitated with ammonium sulfate then purified on a ERE–Teflon column. The hER was resolved by SDS–gel electrophoresis, electrotransferred to a PVDF membrane and subjected to Western blot analysis. The hER before (lane 1) and after ERE–Teflon purification (lane 2) and a mock infected Sf9 whole cell extract (lane 3) are shown.

^{32}P-labeled hER was purified to near homogeneity by DNA-affinity chromatography and SDS–electrophoresis (Fig. 3). We have also purified, using the same procedure, the ^{32}P-labeled hER from MCF-7 cells, after adding the recombinant hER as a non-labeled carrier protein.

Western blot analysis of the ERE–Teflon purified hER

We and others have shown that phosphorylation of steroid hormone receptors correlates with their affinity for specific DNA sequences [17, 21]. Since purification of the hER involved DNA-affinity chromatography we sought to examine whether preferential selection for one form of the receptor occurred (e.g. the hyperphosphorylated form). Western blot analysis of the hER purified by DNA-affinity revealed that the ratio of the 66 and 67 kDa forms of the hER was approx. 1:1 both before and after purification (Fig. 4). These results are not incompatible with earlier findings that showed receptor phosphorylation may modulate specific DNA binding [21]. The ERE–Teflon purification of the hER is a bulk procedure in which approx. 1 mg of hER is loaded onto the ERE–Teflon column followed by stepwise salt elution. In addition, the ERE–Teflon column's binding capacity (*ca* 250 nmol) greatly

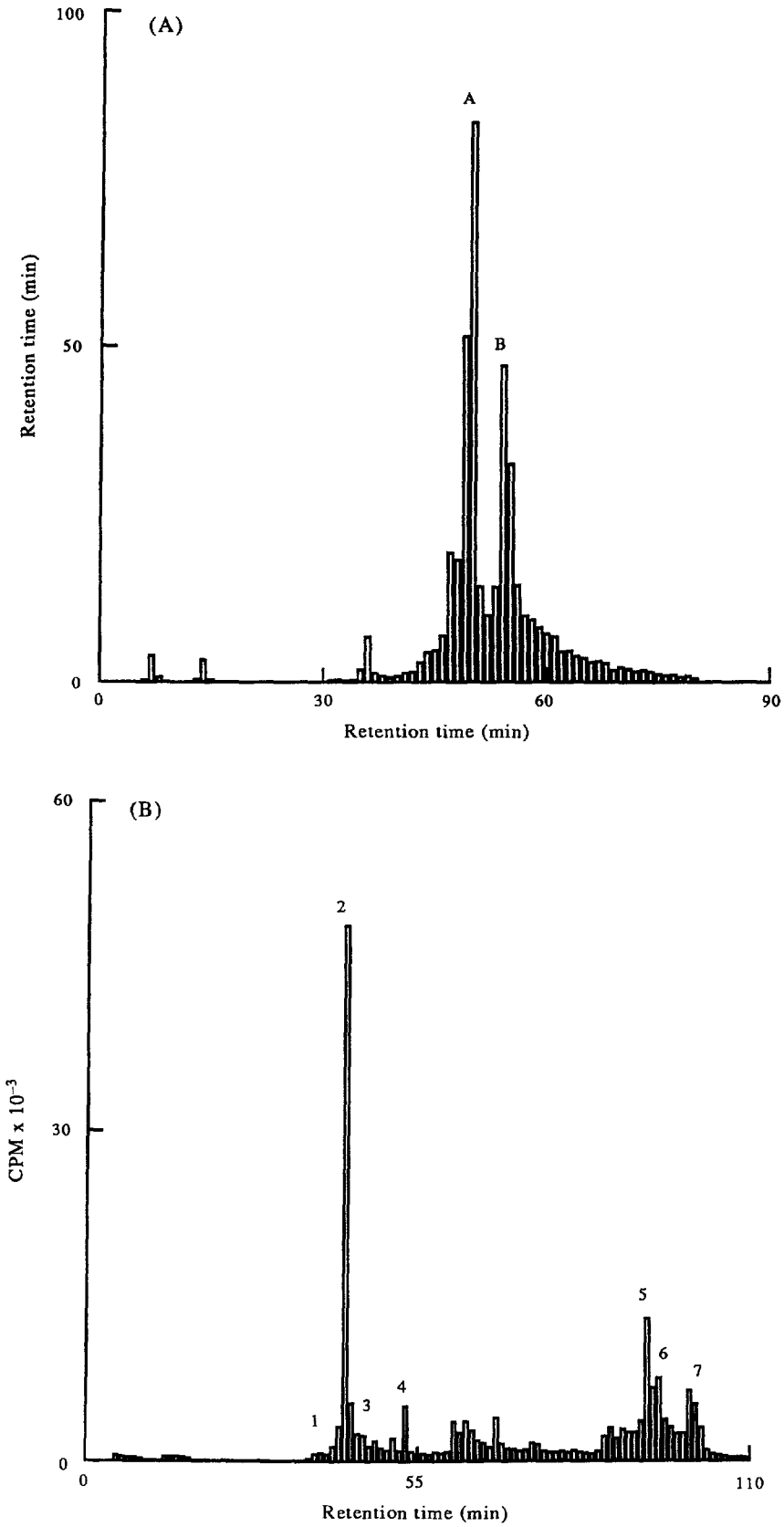

Fig. 5(A,B)—*legend opposite.*

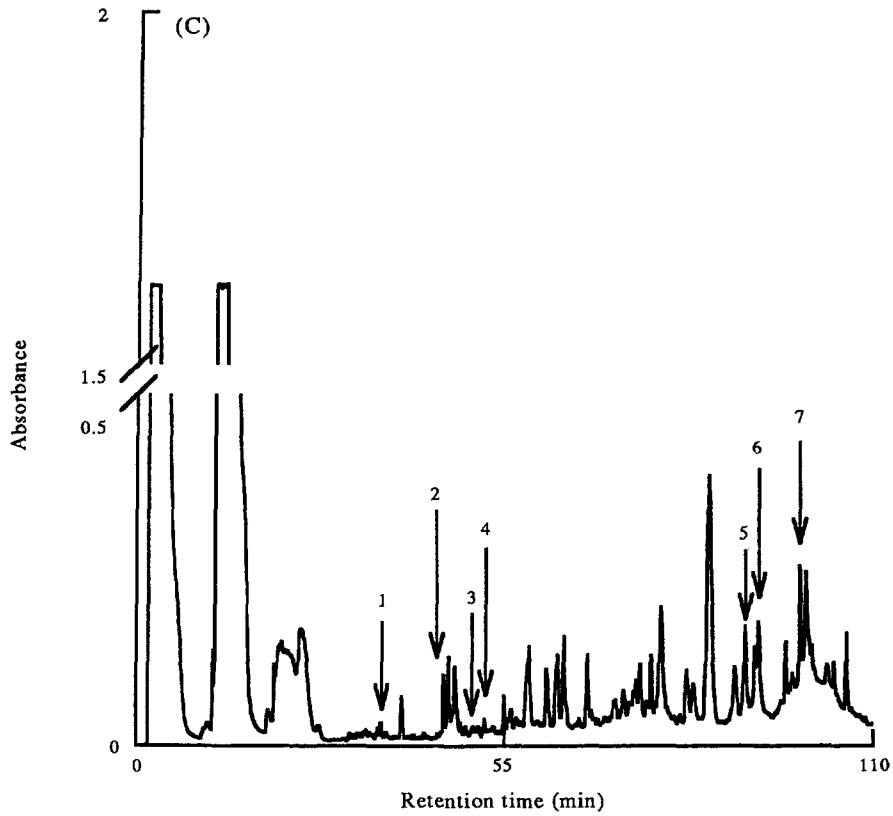

Fig. 5. The RP-HPLC of the [^{32}P]phosphopeptides of the recombinant hER. Purified hER from Sf9 cells labeled with [^{32}P]orthophosphate for 4 h followed by 30 min with 1 μM estradiol. The ^{32}P-labeled hER was digested with CNBr and trypsin. (A) The [^{32}P]phosphopeptides of the recombinant hER were separated after CNBr digestion on a C 4 column. (B) Peaks A and B were combined and digested with trypsin, then separated with a C 18 column. (C) The UV profile of the CNBr/tryptic digest at 220 nm is shown.

exceeds the receptor concentration. Therefore, it is not surprising that under these conditions a difference in DNA binding between the 66 and 67 kDa forms was not detected.

The RP-HPLC of the [^{32}P]phosphopeptides from the recombinant hER

Sf9 cells infected with the AcNPV–hER baculovirus were incubated with [^{32}P]phosphate for 4 h followed by 30 min with estradiol. Thereafter, the ^{32}P-labeled recombinant hER was purified and digested with CNBr. Trypsin used alone failed to give consistent and complete cleavage of the receptor. The separation of the CNBr peptides prior to trypsin digestion also allowed for an initial purification of the [^{32}P]phosphopeptides from unlabeled peptides. However, due to the tendency of the large CNBr peptides to aggregate their separation was poor. The CNBr peptides of the recombinant hER were resolved into two major ^{32}P-labeled peaks, A and B, by RP-HPLC using a C4 column. Peak A of the CNBr digest had a retention time of 50 min and 54% of the radioactivity, while peak B had a retention time of 54 min and 36% of the radioactivity [Fig. 5(A)]. The [^{32}P]phosphopeptides were injected onto the reverse phase columns in two aliquots, thus two solvent fronts [Fig. 5(C)] and two peaks of free [^{32}P]phosphate at 7 and 14 min [Figs 5(A), 6 and 7] were observed and do not represent phosphopeptides.

The [^{32}P]phosphopeptides in peaks A and B were pooled, digested with trypsin and separated by RP-HPLC on a C 18 column. Five [^{32}P]phosphopeptides were consistently resolved in six separate experiments, these were: [^{32}P]phosphopeptides 2, 4, 5, 6 and 7 [Fig. 5(B)]. Redigestion of [^{32}P]phosphopeptides 2, 4, 5, 6 and 7 with trypsin yielded the identical [^{32}P]phosphopeptide profiles indicating complete digestion of the hER was achieved. [^{32}P]Phosphopeptide 2 or serine 167, which consistently eluted at 44 min, incorporated the most [^{32}P]phosphate of the five [^{32}P]phosphopeptides, accounting for 50% of the total radioactivity (Table 1) [26]. The UV profile of the CNBr/tryptic digest demonstrates that every [^{32}P]phosphopeptide corresponded to a UV peak [Fig. 5(C)]. In addition, most of the [^{32}P]phosphopeptide UV peaks contained 100–300 pmol of [^{32}P]phosphopeptide.

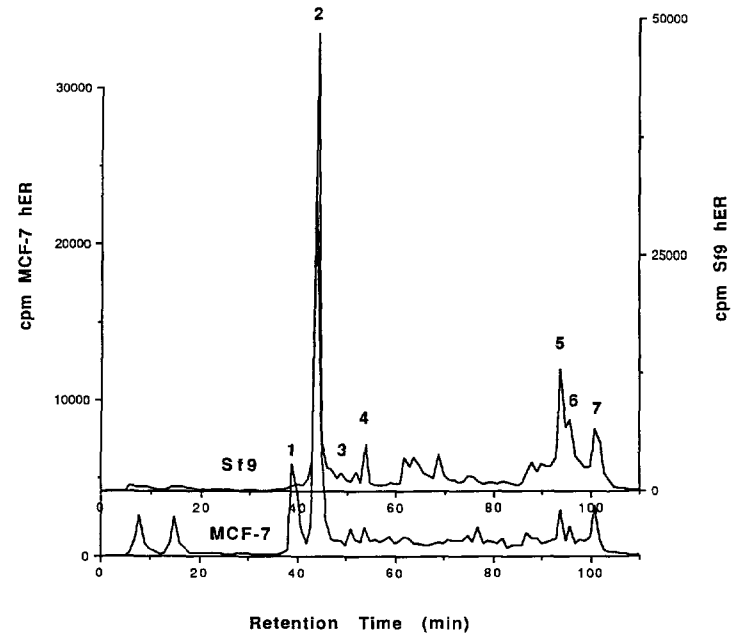

Fig. 6. Comparison of the [^{32}P]phosphopeptide maps of the hER from MCF-7 and Sf9 cells. Purified ^{32}P-labeled hER from estradiol-treated MCF-7 and estradiol-treated Sf9 cells were digested with CNBr followed by trypsin. The [^{32}P]phosphopeptides were then separated by RP-HPLC with a C 18 column.

The RP-HPLC of the hER [^{32}P]phosphopeptides from MCF-7 cells treated with estradiol

The CNBr/trypsin digest of the hER from MCF-7 cells revealed six specific [^{32}P]phosphopeptides. The CNBr digest of the native hER showed two major ^{32}P-labeled peaks, similar to that of the ^{32}P-labeled recombinant hER (data not shown). The RP-HPLC revealed that the tryptic digest of the hER from MC-7 cells treated with estradiol showed six [^{32}P]phosphopeptides 1, 2, 4, 5, 6 and 7. The [^{32}P]phosphopeptides 2, 4, 5, 6 and 7 from hER produced either in Sf9 or MCF-7 cells exhibited identical retention times and are therefore conserved [^{32}P]phosphopeptides (Fig. 6 and Table 1). As observed with the recombinant hER, [^{32}P]phosphopeptide 2 or serine 167 of the native hER was the predominant [^{32}P]phosphopeptide, accounting for 43%

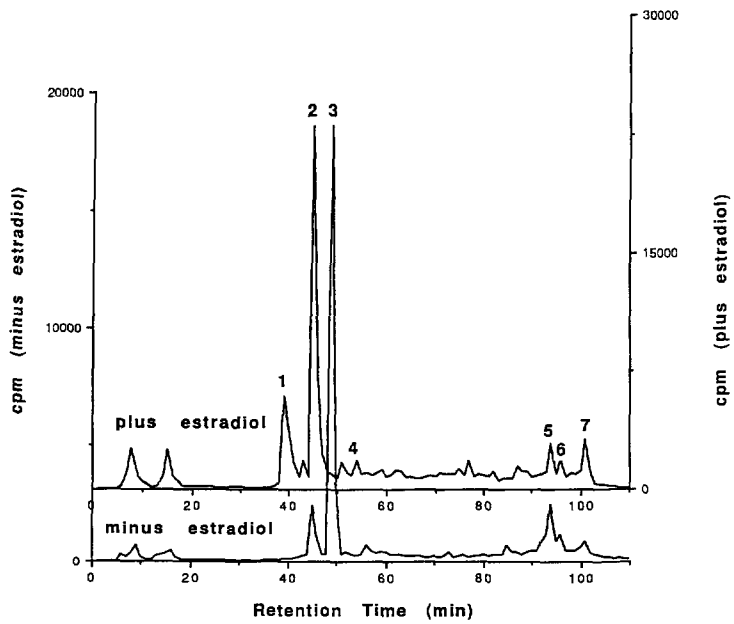

Fig. 7. Estradiol-induced site-specific phosphorylation of the hER in MCF-7 cells. Purified and ^{32}P-labeled hER was isolated from MCF-7 cells that were either, not-treated (minus estradiol) or treated with estradiol (plus estradiol). The hERs were digested with CNBr followed by trypsin and then separated by RP-HPLC with a C 18 column.

Table 1. Comparison of the distribution of the [^{32}P]phosphate in the phosphopeptides of the hER from estradiol-treated Sf9 and MCF-7 cells

Phosphopeptide number	% of [^{32}P] in each of the phosphopeptide of the hER from Sf9 cells	% of [^{32}P] in each of the phosphopeptide of the hER from MCF-7 cells
1	1.0	15.0
2	50.0	43.0
3	0.8	2.3
4	4.0	2.5
5	15.0	6.7
6	12.0	5.0
7	11.5	7.0

The percentages shown are based upon the fraction of the total radioactivity recovered from the C 18 reverse phase column. The values shown for the Sf9 cells are the means of six separate experiments and two separate experiments for the MCF-7 cells.

of the [^{32}P]phosphate incorporated into the receptor [26].

[^{32}P]Phosphopeptide 1 is unique to the hER from MCF-7 cells since it was not detected in the recombinant hER (Fig. 6). [^{32}P]Phosphopeptide 7, whether from Sf9 or MCF-7 cells, incorporated a similar percentage of [^{32}P]phosphate into the hER. Whereas, [^{32}P]phosphopeptides 4, 5 and 6 of the hER from Sf9 cells had incorporated more [^{32}P]phosphate than the hER from MCF-7 cells (Table 1).

The RP-HPLC of the hER [^{32}P]phosphopeptide from MCF-7 cells not treated with estradiol

The hER from MCF-7 cells, not treated with estradiol, was purified and digested with CNBr. The RP-HPLC resolution of the CNBr digestion showed two [^{32}P]phosphopeptide peaks which had identical retention times as the hER from estradiol-treated MCF-7 cells (data not shown). The tryptic digest of the CNBr-digested hER showed six [^{32}P]phosphopeptides 2, 3, 4, 5, 6 and 7. Note that [^{32}P]phosphopeptide 1 is greatly diminished and is therefore unique to the hER from MCF-7 cells treated with estradiol (Fig. 7). [^{32}P]Phosphopeptide 3, consistently eluted at 49 min, is unique to the hER in the absence of estradiol, accounting for 65% of the radioactivity incorporated into the receptor. Therefore, the phosphorylation site(s) on [^{32}P]phosphopeptide 3 appear to undergo a dephosphorylation after estradiol binding. Fractions 35–52 min, i.e. [^{32}P]phosphopeptide peaks 1, 2, and 3 of the hERs, from the estradiol-treated and nontreated MCF-7 cells were pooled, dried and redigested with trypsin. The RP-HPLC showed that [^{32}P]phosphopeptides 1, 2 and 3 still eluted as three distinct phosphopeptides at 37, 44 and 49 min, respectively (data not shown).

Comparison of the [^{32}P]phosphate incorporated into the [^{32}P]phosphopeptides of the hER from the non-treated and estradiol-treated MCF-7 cells revealed that three [^{32}P]phosphopeptides, i.e. [^{32}P]phosphopeptide 1, 2 and 4 were hyperphosphorylated after estradiol treatment (Table 2). [^{32}P]Phosphopeptide 1 underwent the greatest percentage change after estradiol treatment, showing a 50-fold increase, while [^{32}P]phosphopeptide 4 increased 16-fold. Although [^{32}P]phosphopeptide 2 or serine 167 showed only a 4-fold increase in response to hormone it nevertheless incorporated the majority of the [^{32}P]phosphate and was the dominant [^{32}P]phosphopeptide.

Phosphorylation of the recombinant hER by DNA-PK

To more directly define the mechanism of phosphorylation of the hER we investigated the effects of the DNA-PK on the hER. The recombinant hER was purified without phosphatase inhibitors, then the hER was incubated with [γ-^{32}P]ATP, DNA-PK and either DNA or a 26 bp ERE. The products were TCA precipitated and analyzed by SDS–gel electrophoresis and then transferred to a PVDF membrane. Autoradiography of the membrane revealed that [^{32}P]phosphate was incorporated into the recombinant hER only in the presence of DNA-PK and DNA or an ERE, but not in the absence of DNA [Fig. 8(A)]. Interestingly, the DNA-PK also phosphorylated casein kinase II a contaminant in our purified recombinant hER preparation [Fig. 8(A)] [26]. Western blot analysis with an anti-hER antibody of the purified recombinant hER in the absence of DNA-PK (data not shown) and in the presence of DNA-PK but without DNA revealed only a 66 kDa hER band [Fig. 8(B)]. Phosphorylation of the recombinant hER by DNA-PK protein kinase and an ERE or DNA elicited a conformational change in the receptor resulting in a second slower migrating form at 67 kDa, in addition to the 66 kDa band [Fig. 8(B)]. The two hER forms produced by phosphorylation with the DNA-PK migrate identically to the two hER forms induced after estrogen treatment of MCF-7 or Sf9 cells (Fig. 2).

In vitro assays with the recombinant hER and protein kinases A and C

Numerous reports have demonstrated that cells treated with activators of protein kinases A and C (PKA and PKC) result in the hyperphosphorylation

Table 2. Comparison of the distribution of the [^{32}P]phosphate in the phosphopeptides of the hER from non-estradiol and estradiol-treated MCF-7 cells

Phosphopeptide number	% in the absence of estradiol	% in the presence of estradiol	Fold change
1	0.3	15.0	50.0
2	10.0	43.0	4.3
3	65.0	2.3	(−28.0)
4	0.15	2.5	16.0
5	8.0	6.7	1.0
6	6.0	5.0	1.0
7	3.5	7.0	2.0

Each determination is the mean of two separate experiments.

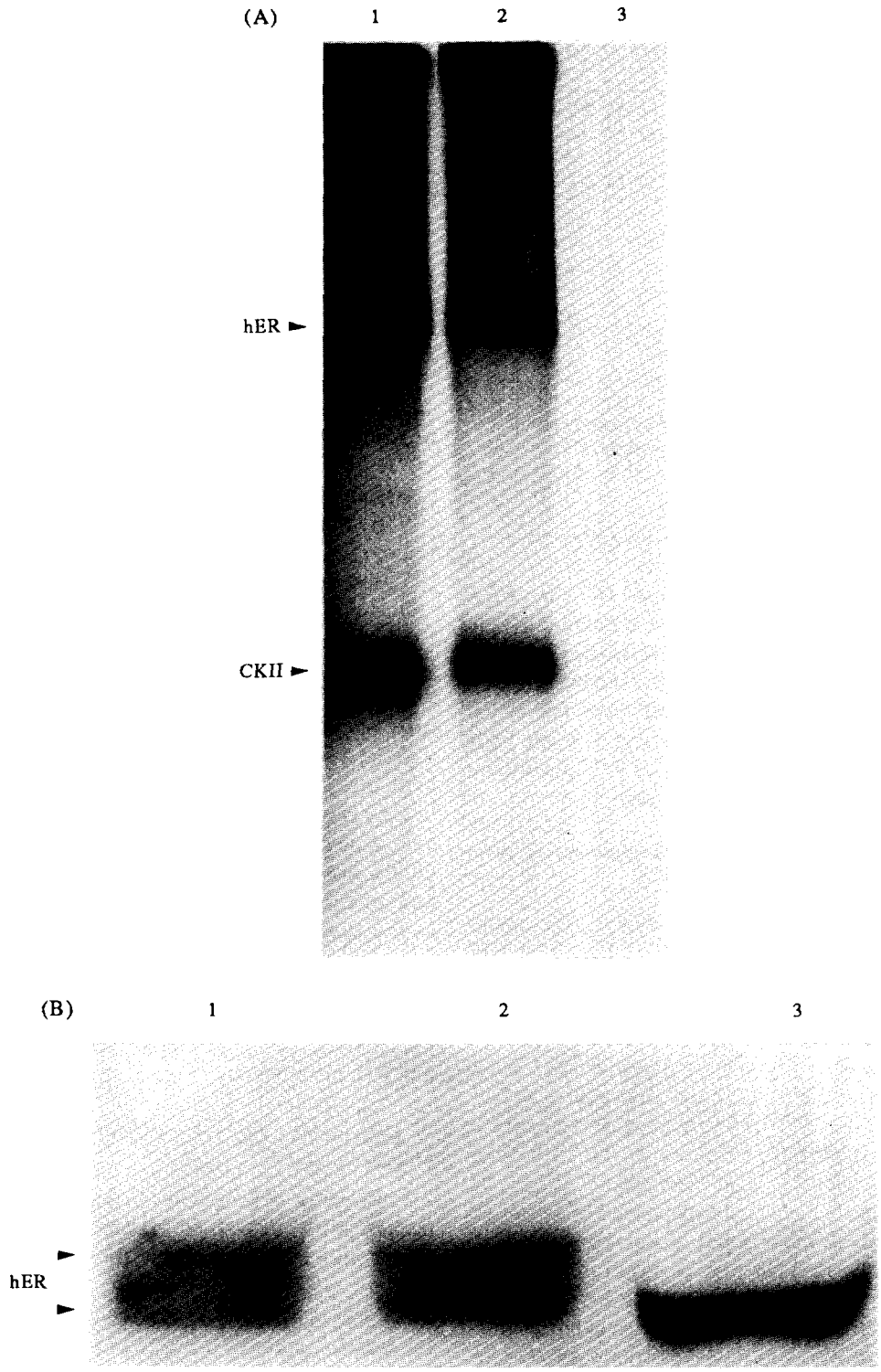

Fig. 8. *In vitro* phosphorylation of the purified recombinant hER by DNA-PK. The purified recombinant hER was incubated with [γ-^{32}P]ATP and DNA-PK, the products were TCA precipitated, separated by SDS-gel electrophoresis and transferred to a PVDF membrane. (A) Autoradiography of the PVDF membrane; (B) Western blot with an anti-hER antibody. The purified recombinant hER with the DNA-PK and the ERE (lane 1), with the DNA-PK and ssDNA (lane 2) and with the DNA-PK (lane 3).

and increased transcriptional activity of steroid hormone receptors [15, 16]. To test this hypothesis directly we have incubated the purified recombinant hER with [γ-^{32}P]ATP and PKA or PKC. The products were analyzed by SDS-gel electrophoresis and transferred to a PVDF membrane for autoradiography. Incubation of the hER with either PKA or PKC did not result in [^{32}P]phosphate incorporation into the receptor *in vitro* (Fig. 9). Interestingly, the buffer used for the PKA assay facilitated background [^{32}P]phosphate

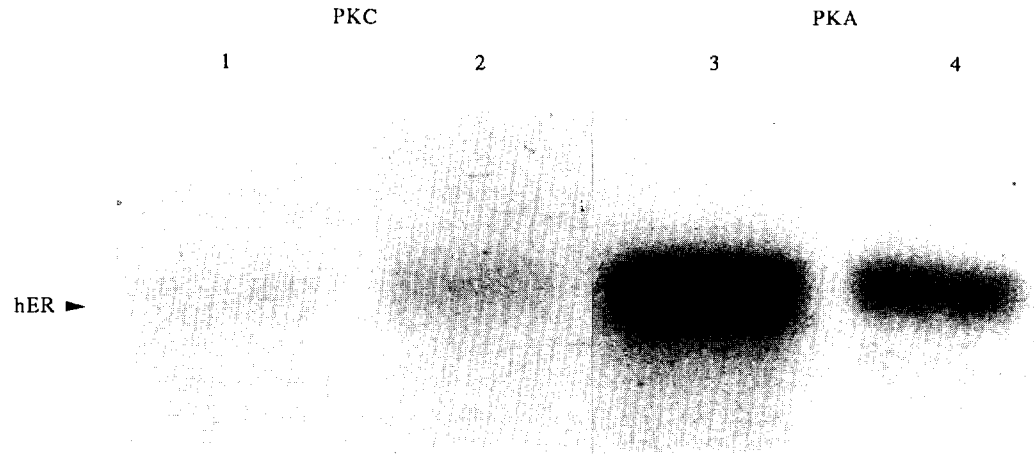

Fig. 9. *In vitro* incubation of the purified recombinant hER with PKA or PKC. The purified recombinant hER was incubated with [γ-^{32}P]ATP and PKA or PKC, the products were TCA precipitated, separated by SDS–gel electrophoresis and transferred to a PVDF membrane. The purified recombinant hER (lane 1 and 3), with PKC (lane 2), and PKA (lane 4).

incorporation into the hER probably by a contaminating protein kinase, such as casein kinase II present in our purified hER preparation [26]. Addition of PKA to the hER slightly reduced the [^{32}P]phosphate incorporated into the receptor (Fig. 9). The contaminant casein kinase II was not activated under the conditions used in the PKC assay and therefore almost no [^{32}P]phosphate incorporation was detected. Different concentrations of PKA, PKC and ATP were tested, and no [^{32}P]phosphate incorporation into the hER was observed. The lack of [^{32}P]phosphate incorporation into the hER was not due to a lack of activity by either protein kinase as they phosphorylated known peptide substrates (data not shown).

DISCUSSION

Determining the molecular consequences of estrogen receptor phosphorylation requires the identification of the specific sites of phosphorylation and the protein kinases involved. To this end, we have employed a recombinant baculovirus to overexpress the hER and investigate the receptor's phosphorylation. The large quantity of hER produced by a baculovirus overexpression system has made the purification of ^{32}P-labeled hER and its [^{32}P]phosphopeptides feasible. We found that DNA-affinity chromatography with an ERE–Teflon matrix followed by SDS–gel electrophoresis and a CNBr and tryptic digest of the purified hER gave reproducible [^{32}P]phosphopeptide and UV absorbance chromatographic profiles.

The recombinant hER expressed in the estradiol-treated Sf9 insect cells is phosphorylated with remarkable similarity to the native hER in MCF-7 cells. Consistent with this observation is our previous finding that the recombinant hER was full length, completely soluble and biologically active with respect to hormone- and DNA-binding [25]. In some instances, the phosphorylation of proteins overexpressed in Sf9 cells has been shown to be nearly identical to that in mammalian cell lines. For example, SV40 T-antigen [31] and the retinoblastoma protein [32] exhibit almost identical phosphopeptide maps whether they are produced in insect or mammalian cell lines. [^{32}P]Phosphopeptides 2, 4, 5, 6 and 7 from the hER were found to be conserved between the Sf9 and MCF-7 cell lines. The high degree of conservation of the [^{32}P]phosphopeptides from the hER derived from the MCF-7 or Sf9 cells suggest that the protein kinases involved may be conserved from invertebrate to vertebrates. [^{32}P]Phosphopeptides 1 and 3 appear to be unique to the hER from MCF-7 cells. The hER, whether from estradiol treated MCF-7 or Sf9 cells, showed one dominant site of phosphorylation: [^{32}P]phosphopeptide 2 or serine 167 [26].

In our previous study [17] the MCF-7 cells were incubated with the [^{32}P]orthophosphate for 4 h, a short time relative to the half-life of the hER which is approx. 3–5 h [33]. Consequently, estrogen stimulated phosphorylation was detected, however, basally phosphorylated sites may not necessarily have been detected. In this study we labeled the hER in the MCF-7 cells for 16 h with [^{32}P]orthophosphate which would presumably be at or near steady state synthesis of the hER. Thus, comparison of the estradiol-treated and non-treated MCF-7 cells following long-term [^{32}P]orthophosphate labeling surprisingly revealed that the unliganded hER contains a major [^{32}P]phosphopeptide, [^{32}P]phosphopeptide 3, that undergoes dephosphorylation after estradiol binding. At present we have not labeled the hER in Sf9 cells with [^{32}P]orthophosphate in the absence of estradiol to determine if [^{32}P]phosphopeptide 3 is present. These results are in agreement with Orti *et al.* [34] who show that the glucocorticoid receptor may also be regulated by both phosphorylation and dephosphorylation after hormone binding.

One function of the phosphorylation on the [^{32}P]phosphopeptide 3 site(s), which is present only on the unliganded receptor, may be to repress receptor action prior to hormone binding. For example, phosphorylation of the yeast ADR1 gene product inhibits its transcriptional activity [35], phosphorylation also represses specific DNA binding by c-Myb [36] and myogenein [37]. Perhaps the dephosphorylation/phosphorylation we observe after hormone binding is similar to the mitogen activation of c-Jun. In response to growth factors, c-Jun is dephosphorylated on sites that inhibit DNA binding and phosphorylated on other sites that promote transcriptional activation of the protein [38, 39]. Recently, intriguing data have suggested that the phosphorylation of two steroid receptors, vitamin D receptor and NGFI-B, in or near the DNA binding domain inhibits DNA binding [40, 41]. The phosphorylation of the vitamin D receptor by protein kinase C *in vitro* and the subsequent loss of DNA binding is very provocative since serine 51, phosphorylated by protein kinase C, is conserved within the thyroid/retinoic acid and estrogen receptor families [40]. Serine 212 of the hER is analogous to serine 51 of the vitamin D receptor, both of which are located between the zinc fingers in the DNA binding domain.

An alternative explanation is that the phosphorylation on the phosphopeptide 3 site(s) may enhance an activity of the receptor prior to hormone binding, such as nuclear translocation or protein interactions, e.g. heat shock proteins [42]. The yeast transcription factor SW15 undergoes nuclear entry only after a cell cycle dependent dephosphorylation of three serine residues [43].

We have previously shown that *in vitro* casein kinase II specifically phosphorylates the hER on serine 167, the major estradiol-induced phosphorylation site on the receptor [26]. The present report describes the identification of the DNA-PK as another regulator of the phosphorylation of the hER. The DNA-PK incorporated [^{32}P]phosphate into the hER produced a 67 kDa form of the receptor that displayed a slower electrophoretic mobility. The 67 kDa form is significant since this form of the hER correlates with the 67 kDa form produced after estradiol treatment of MCF-7 cells. Weigel *et al.* [44] have previously reported that the DNA-PK phosphorylated the chicken progesterone receptor and altered the SDS-mobility of the receptor. Other evidence with the progesterone receptor suggests that the phosphorylation occurs in two stages: a hormone-dependent phosphorylation and then a DNA-dependent phosphorylation [29]. Recently, Lahooti *et al.* [45] also claimed that the estrogen receptor is phosphorylated after hormone- and DNA-binding. Therefore, the evidence suggests that the DNA-PK may phosphorylate the hER *in vivo*.

Recently, much attention has been focused on the ligand-independent activation of steroid hormone receptors. Numerous groups report that activators of PKA and PKC result in the hyperphosphorylation and transcriptional activity of the hER [15, 16]. We have not been able to directly phosphorylate the purified recombinant hER with PKA or PKC *in vitro*. The hER contains several consensus sequences for PKA and PKC. Therefore, at least *in vitro*, these sites are obscured or not recognized by the kinases. The intracellular actions of PKA and PKC probably only indirectly result in the phosphorylation and transcriptional activity of the hER. For example, PKA or PKC may activate through phosphorylation other protein kinases and/or transcriptional activators which may directly act on the hER.

In conclusion, the large quantity of hER produced by the baculovirus overexpression system has allowed identification of the [^{32}P]phosphopeptides, and eventually the receptor's phosphorylation sites, from the native and recombinant hER. Long-term [^{32}P]orthophosphate-labeling of MCF-7 cells followed by estradiol treatment has shown that phosphorylation and a dephosphorylation of the hER is occurring. In addition, the hER is phosphorylation by DNA-PK, but not by PKA or PKC.

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