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Identification of Phosphorylation Sites in the Mouse Oestrogen Receptor

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Phosphorylation sites in the mouse oestrogen receptor, expressed in COS-1 cells in the presence of 17β-oestradiol, have been mapped by solid phase microsequencing. The receptor was first radiolabelled with [^{32}P]orthophosphate and a number of ^{3}H- or ^{14}C-labelled amino acids, immunopurified and then tryptic peptides were separated by thin layer chromatography or high performance liquid chromatography. Amino acid sequence analysis indicated that Ser-122, Ser-156, Ser-158 and Ser-298 were phosphorylated. The substitution of Ser-122 and Ser-298 with alanine had a negligible effect on the transcriptional activity of the receptor in transfected cells. However, a reduction of transcriptional activity was observed when Ser-122 was mutated in the context of mutations in a putative amphipathic α-helix involved in AF-2 activity. Thus a region of AF-1 that encompasses Ser-122 appears to interact with AF-2 in the full-length receptor.

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INTRODUCTION

The oestrogen receptor is a member of a superfamily of nuclear receptors most of which appear to be phosphoproteins [1]. While phosphorylation of steroid receptors is increased by hormone binding it has been found to occur in stages, at least for the progesterone and oestrogen receptors [2–6]. In addition to basal phosphorylation there is an increase in phosphorylation upon hormone binding and a further increase upon DNA binding. In view of the conservation of the domain structure of nuclear receptors [7–10], it is possible that the role of phosphorylation is also conserved. To date, phosphorylation has been implicated in a number of different functions including receptor processing and shuttling [1, 11], DNA binding [12] and transcriptional activation [13–16].

To investigate the role of phosphorylation in the action of the mouse oestrogen receptor we have mapped phosphorylation sites by solid phase microsequencing of phosphopeptides. This approach was used to identify phosphorylation sites in the mouse glucocorticoid receptor [17] but has not been reported for other receptors. We have previously shown by two dimensional phosphopeptide mapping that the sites of phosphorylation in the oestrogen receptor were similar irrespective of the ligand bound [4]. In view of this, receptors were expressed in COS cells in the presence of 17β-oestradiol since hormone binding increased the total amount of phosphorylation and facilitated the microsequencing. We then investigated the transcriptional activity of a number of mutant receptors in transient transfection experiments.

MATERIALS AND METHODS

DNA clones

Mutations were introduced into the coding sequence of the mouse oestrogen receptor cDNA [18] in order to replace either the serine residue at positions 122 or 298 using polymerase chain reactions. To mutate Ser-122, oligonucleotide primers were synthesized corresponding to the sequence 5′ to the Not I site at position 271 (Primer 1) and 3′ to the BsmI site at position 646 (Primer 4). A pair of complementary oligonucleotides (Primer 2 and Primer 3) was synthesized in which the nucleotides coding for Ser-122 were altered to code for alanine, a NheI site introduced and the PvuII site at position 359 destroyed. The sequences of the four primers is as follows: Primer 1,

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CAGTCGGGCATCGCC; Primer 2, CCGCAGCTAGCACCTTTCCTGCACCCG; Primer 3, GAAAGGTGCTAGCTGCGGCGGCG; Primer 4, GGACACATGTAGTCATTG. To replace Ser-298 with alanine, primers were synthesized corresponding to the sequence 5′ to the Bsm I site at position 646 (Primer 1) and 3′ to the XbaI site at position 1010 (Primer 4). A pair of complementary oligonucleotides (Primer 2 and Primer 3) were synthesized in which the nucleotides coding for Ser-298 were altered to code for alanine. The sequences of the four primers are as follows: Primer 1, GCGAAGGCTGCAAGG; Primer 2, TGGCCAGCTCCTCTTGTGATTAAGCA; Primer 3, TCACAAGAGGAGCTGGCCAAAGGTTGGCA; Primer 4, TAAGCCCATCATTGAGG. The introduction of mutations was confirmed by DNA sequencing.

Cell culture and transient transfection experiments

COS-1 and NIH3T3 cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) foetal calf serum (GIBCO). For transfection experiments, COS-1 cells were harvested at 70% confluency and, after suspension in phosphate-buffered saline (PBS), 2×10^7 cells per ml were transfected by electroporation with 20 μg MOR 1-599, MOR S-122A or MOR S-298A. Electroporation was performed at 450 V and 250 μFarad using a Bio-rad Gene pulser as previously described [19]. Electroporated cells were grown in DMEM without phenol red supplemented with 10% (v/v) dextran-charcoal treated (DCC) foetal calf serum. NIH3T3 cells were plated out at 2×10^5 cells/6 cm dish in phenol red-free DMEM, 10% dextran-charcoal treated foetal calf serum and transfected using the calcium phosphate coprecipitation method as previously described [20]. Transfected DNA included a reporter plasmid pEREBLCAT (5 μg), 1 μg of an internal control plasmid (pJ3 luciferase), the appropriate receptor expression vector as indicated in the Results (0.5 μg) and pJ3Ω [21] to a total of 10 μg/dish. Following transfection, cells were maintained in the absence or presence of 10^{-8} M oestradiol. After 48 h the cells were harvested and extracts assayed for luciferase [22] and CAT activity [23]. Luciferase activity was used to correct for the differences in transfection efficiency in all experiments.

Radiolabelling of the oestrogen receptor

COS-1 cells, transfected with MOR1-599, were washed in phenol red-free culture medium lacking either phosphate and/or the amino acids leucine, methionine, valine or tyrosine as indicated. The culture medium was then supplemented with 500 μCi/ml [^{32}P]orthophosphate alone or in combination with either 500 μCi/ml L-[4,5-^{3}H]leucine (sp. act. 147 Ci/ml), 100 μCi/ml L-[^{35}S]methionine (specific activity 1140 Ci/ml, NEN), 500 μCi/ml L-[3,4-^{3}H]valine (sp. act. 30 Ci/ml) or 25 μCi/ml L-[^{14}C]tyrosine (sp. act. 475 Ci/ml). All radioisotopes were purchased from Amersham, U.K. Cells were collected [4] and whole cell extracts prepared as previously described [24] except that the buffer also contained 100 μM sodium vanadate, 20 mM sodium fluoride, 20 mM β-glycerol phosphate and 10 mM sodium molybdate. The protein concentration of each extract was determined with a Bio-Rad protein assay reagent.

Immunoprecipitation of oestrogen receptor

Aliquots of whole cell extracts (300 μg) were incubated with the human oestrogen receptor monoclonal antibody H222 (1 μg) at 4°C. After 18 h goat anti-rat IgG (Dakopatts, Glostrup, Denmark) was added as bridging antibody and left at 4°C for 1 h. The extracts were then diluted with a low salt NET buffer containing protease and phosphatase inhibitors (150 mM NaCl, 0.1% NP-40, 3 mM EDTA, 1 mM DTT, 50 mM Tris–Cl (pH 7.5), 0.25% gelatin, 0.02% sodium azide, 0.5 mg/ml bacitracin, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin, 100 μM sodium vanadate, 20 mM sodium fluoride, 2 mM β-glycerol phosphate and 10 mM sodium molybdate). The receptor–antibody complexes were then isolated using protein A-Sepharose (Pharmacia) as previously described [4]. The radiolabelled receptors were resuspended in SDS–PAGE loading buffer and analyzed on SDS–10% polyacrylamide gels.

Two dimensional electrophoresis and chromatography

Tryptic phosphopeptide maps of the oestrogen receptor were obtained using the method of Boyle *et al.* [25]. Briefly the immunoprecipitated receptors were digested with 10 μg TPCK-treated trypsin (Worthington) in 500 μl ammonium bicarbonate pH 8.0 at 37°C for 18 h. The digested receptor protein was then lyophilized and reconstituted in 10 μl of electrophoresis buffer. About 700 cpm of the digested peptides were resolved in two dimensions on 14 × 20 cm 100 μm thin-layer cellulose plates (Kodak U.S.A.) Electrophoresis was performed towards cathode at pH 1.9 in formic acid–acetic acid–water [20:80:900] for 30 min at 50 mA followed by ascending chromatography in 1-butanol–acetic acid–pyridine–water [75:15:50:60].

Reverse phase HPLC analysis

The trypsin digested phosphorylated receptor protein was lyophilized and reconstituted in 50 μl MQ (MilliQ) water. Peptides were chromatographed on a 5 μ 300 Å C8 reverse-phase column (2.1 × 100 mm) equilibrated with 0.05% aqueous trifluoroacetic acid (buffer A) at a flow rate of 0.2 ml min^{-1} and the column maintained at 50°C. Retained peptides were eluted with a 2–30% acetonitrile gradient over 45 min, followed by a 30–80% gradient over 35 min. Fractions were collected at half minute intervals and ^{32}P-radioactivity was determined by Cerenkov counting. The

purified ^{32}P-containing peaks were lyophilized and subjected to solid phase sequencing. The absorbance of eluted material (including the control trypsin samples) was monitored at 220 nm.

Solid phase microsequence analysis

The microsequence analysis of purified phosphopeptides was performed as described by [17]. Briefly, each lyophilized ^{32}P-containing peak was dissolved in 20 μl of 50% acetonitrile (Rathburn Grade S) containing 0.1% trifluoroacetic acid (Pierce), heated to 55°C for 5 min, and covalently attached to a disc of arylamine PVDF (Polyvinylidene difluoride) membrane [26]. After the membrane was moistened with 0.1 M MES, pH 5.0, containing 15% aqueous acetonitrile and 10 mg/ml 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride, the disc was placed in the reaction chamber of a Milli Gen 6600 covalent protein sequenator. The phenylthiohydantoin (PTH) derivative generated during each 34 min Edman cycle was collected so that, after 25 cycles of Edman degradation reaction, less than 1–2% of the original ^{32}P remained on the membrane disc as determined by Cerenkov counting.

Microsequence analysis was also performed on the phosphopeptides separated by electrophoresis and chromatography on TLC plates. Cellulose acetate scrapings of each ^{32}P-labelled peptide were extracted four times with 50% trifluoroacetic acid. The phosphopeptides were freeze-dried and then solubilized in 50% acetonitrile containing 0.1% trifluoroacetic acid and covalently attached to an arylamine derivatized PVDF membrane as described above. The attached phosphopeptides was then subjected to 20 cycles of Edman degradation reactions and the resulting PTH-amino acids were counted for radioactivity.

RESULTS

We have mapped phosphorylation sites in the mouse oestrogen receptor, expressed in COS-1 cells in the presence of 17β-oestradiol, by solid phase microsequencing. Cells were incubated with [^{32}P]orthophosphate and, in some cases, either [^{3}H]leucine, [^{3}H]valine or [^{14}C]tyrosine and then radiolabelled oestrogen receptors were immunoprecipitated with the H222 monoclonal antibody. After purification by SDS–PAGE, the receptor was digested with trypsin and separated on thin layer cellulose (TLC) plates by electrophoresis and chromatography. Autoradiography revealed 8–10 phosphopeptides (Fig. 1) of which 5–6

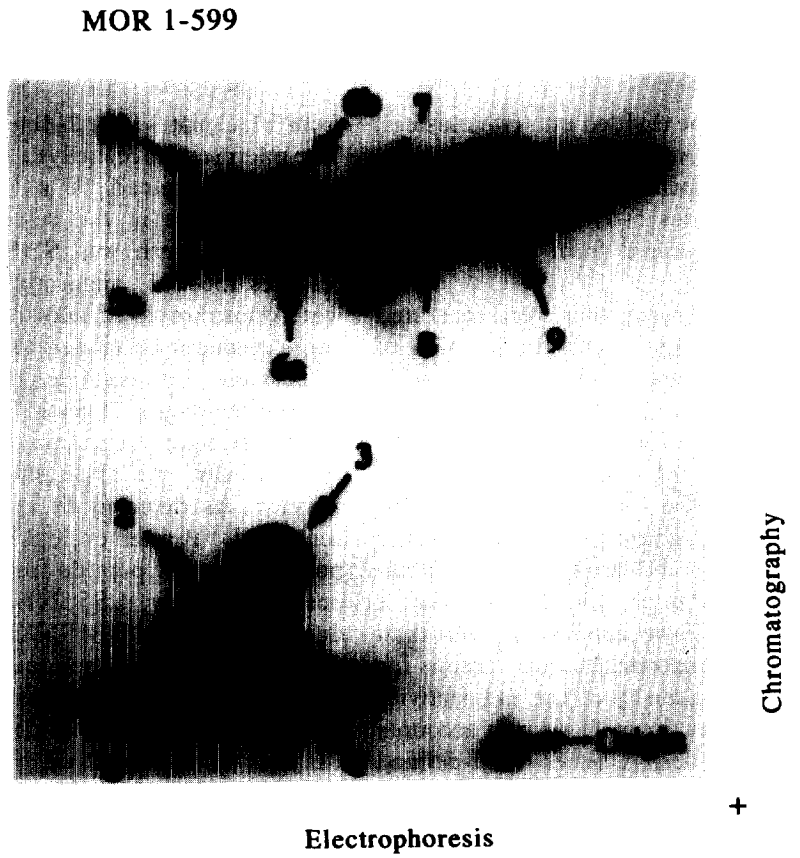


Fig. 1. Two dimensional phosphopeptide map of the wild-type oestrogen receptor. Wild-type oestrogen receptors were labelled with [^{32}P]orthophosphate in the presence of oestradiol and immunoprecipitated with monoclonal antibody H222. Tryptic phosphopeptide map was generated by electrophoresis (towards the cathode) and ascending chromatography and visualized by autoradiography for 5 days. Phosphopeptides were labelled 1–9.

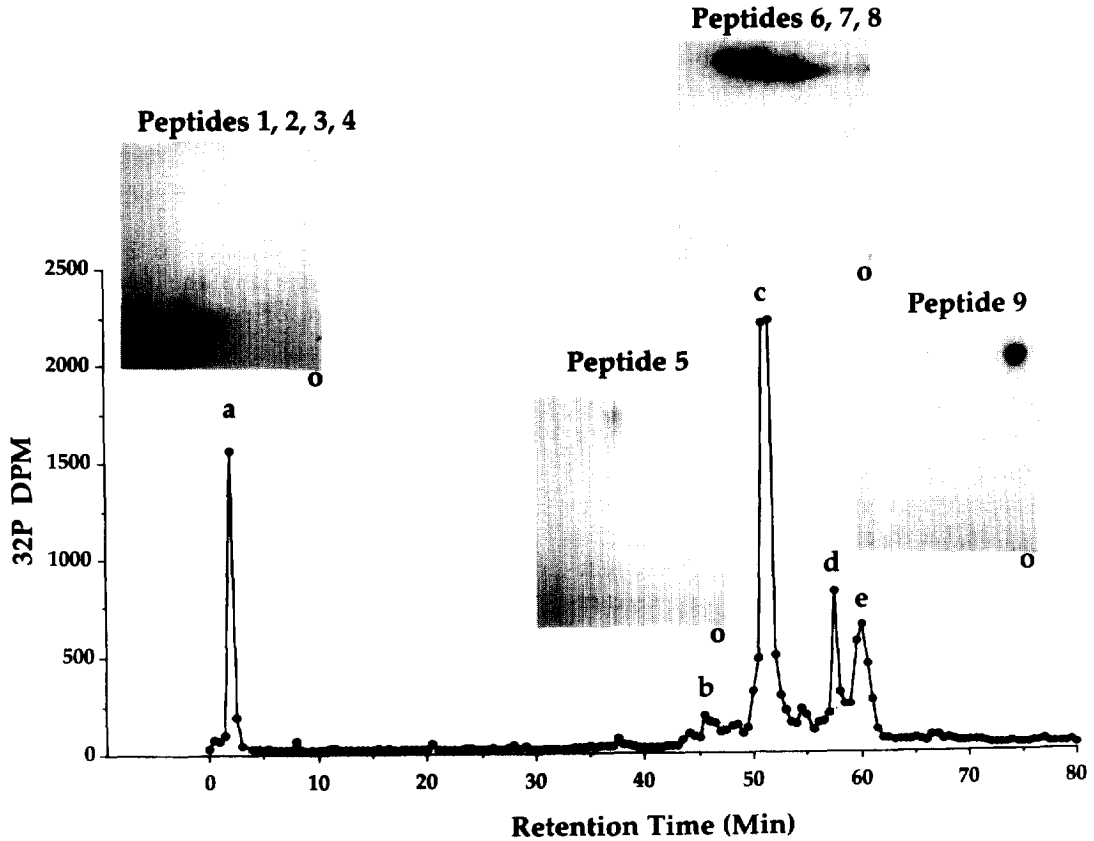


Fig. 2. HPLC analysis of phosphopeptides derived from the oestrogen receptor. The position of phosphopeptides (a–e) was determined by Cerenkov counting and then analysed by two-dimensional phosphopeptide mapping.

were discrete. They could be divided into two clusters, a relatively hydrophilic group of four peptides (peptides 1–4) that migrated towards the cathode and a hydrophobic group (peptides 5–9) that were poorly resolved but could be separated from the origin by ascending chromatography.

After trypsin digestion the peptides were separated by reverse phase HPLC (Fig. 2) and the location of radiolabelled amino acids was determined by solid phase microsequence analysis. The first peak (a), which eluted with very short elution times, was initially presumed to contain free ^{32}P radioactivity but was actually found to contain the four hydrophilic phosphopeptides. The relative amounts of the peptides varied between experiments and, after HPLC chromatography, the amount of ^{32}P in peptides 1 and 4 relative to that in peptides 2 and 3 was consistently less than that found in the starting material but the reasons for this are not known. The majority of the radioactivity which elutes with retention times 45–61 min accounts for the hydrophobic peptides. A series of ill-defined hydrophobic peptides were eluted in peak (c) and additional minor and major hydrophobic peptides were detected in peaks (b), and (e) respectively.

Microsequencing was performed on either the phosphopeptides resolved by HPLC chromatography or, in the case of the four hydrophilic phosphopeptides detected in peak (a), on individual peptides isolated from TLC plates after 2-dimensional peptide mapping. The microsequence analysis showed that peptides 1, 2, and 3 all contained ^{32}P at cycles 1 and 3. The only tryptic peptide in the oestrogen receptor consistent with this pattern was SNSDNR (corresponding to residues 156–161), suggesting that serines 156 and 158 were phosphorylated and that the three radiolabelled peptides might be related to one another (Table 1). It is possible, for example, that as a consequence of incomplete trypsin digestion the peptides 1–3 differ at their C-termini. Variations in trypsin digestion might also account for the differences in their relative amounts, as detected by autoradiography. Peptide 4 contained ^{32}P after cycle 3 (Table 1). There are two potential tryptic peptide fragments with serine at this position, namely, LSSSNEK (corresponding to residues 169–175) and HMSNK (corresponding to residues 520–524). These phosphopeptides are highly charged which accounts for their separation as a hydrophilic cluster and their early elution as a single hydrophilic ^{32}P peak in reverse phase HPLC purification.

The peptide in peak (b) contained ^{32}P at cycle 7 consistent with the sequence of the tryptic peptide, AANLWPSPLVIK (corresponding to residues 292–303). In support, we were able to detect [^{3}H]valine at cycle 10, indicating that serine 298 is a phosphorylation site in the receptor (Table 1). Analysis of peak (c) indicated the presence of ^{32}P at cycle 10 and inspection

of the amino acid sequence of the oestrogen receptor revealed two tryptic peptides (Ser-47, and Ser-198) containing a serine residue in this position. To distinguish these peptides we incorporated [^{3}H]valine and [^{14}C]tyrosine into the receptor and detected these residues as cycles 20 and 22, respectively (Fig. 3). The locations of these residues, however, was not consistent with either Ser-47 or Ser-198 but corresponded with the sequence MLLHPPPQLSPFLHPHGQQVPYYLE between residues 113 and 137. The most likely explanation to account for the generation of this peptide is that cleavage has occurred at a chymotyptic-like site between residues 112 and 113. Chymotyptic activity would also account for the discrepancy in the relative proportion of Tyr-23 and Tyr-24 since this might also represent a potential site. Thus, although trypsin treatment would not be predicted to generate this peptide, the microsequencing data suggests that serine 122 is a major phosphorylation site. Microsequence analysis of peak (d) was inconclusive. Finally the peptide in peak (e) contained ^{32}P at cycle 10. Subsequent analysis of mutant receptors, described below, indicates that this phosphoserine also corresponds to serine 122 and suggests that the peptides in peaks (c) and (e) are related. Thus, in conclusion, solid phase microsequence analysis suggests that Ser-122, Ser-156, Ser-158, Ser-298 and either Ser-171 or Ser-522 are phosphorylation sites in the mouse oestrogen receptor.

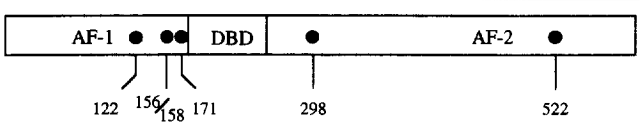
We next substituted a number of the phosphoserine residues with alanines to investigate the phosphorylation and function of the mutant receptors. The phosphorylation pattern of S-122A, determined after transient expression and peptide mapping revealed that the majority of the hydrophobic phosphopeptides generated by the wild-type receptor were not produced by S-122A (Fig. 4). Trace amounts of a phosphopeptide that migrates at a similar position or just ahead of peptide 9 were detected and this probably represents a peptide that is masked when the wild-type receptor is analysed. The amount of peptide 2 was also reduced but, since the relative amounts of peptides 1–3 vary between experiments and were reduced in the wild-type receptor in this analysis, we conclude that phosphorylation of the hydrophilic peptides is unaffected by replacing serine 122 with alanine. Given the microsequencing results, described above, which indicate that phosphoserine was detected in position 10 in the peptides 6–9 isolated in peaks (c) and (e), it seems likely that all four phosphopeptides were phosphorylated at serine 122 but differ in their C-terminal sequences, possibly resulting from incomplete tryptic cleavage. Alternatively but less likely is the possibility that the four peptides contain distinct phosphoserine residues but that their phosphorylation depends on the prior phosphorylation of serine 122.

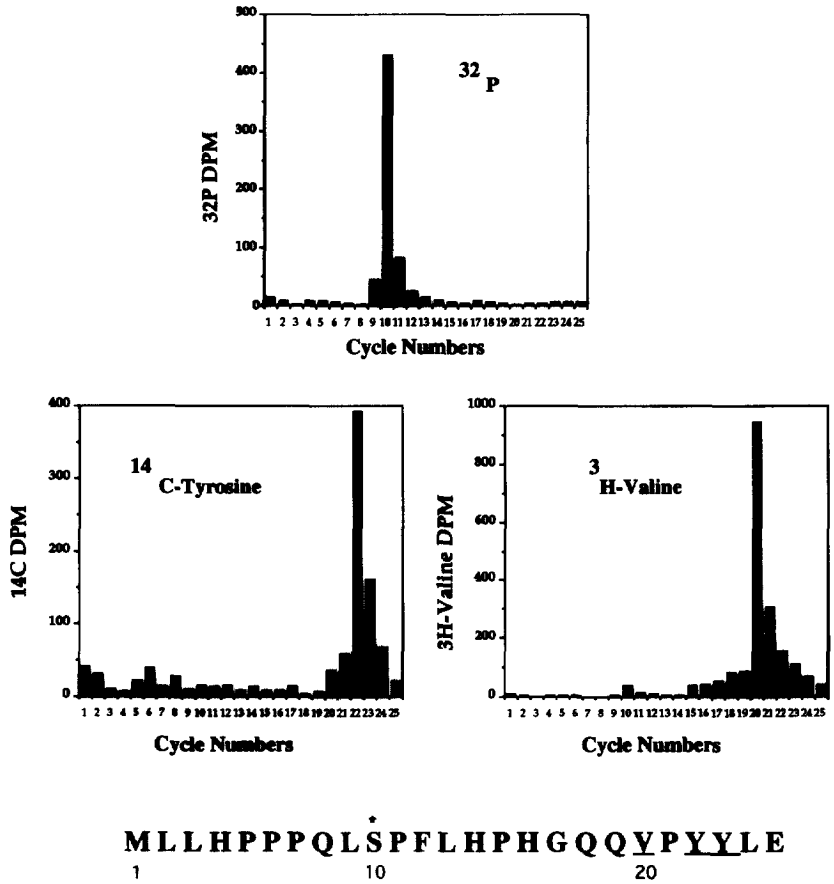
The phosphorylation pattern of S-298A, shown in Fig. 5, indicates that phosphopeptides 5 and 6 were no longer detected but, in addition, the phosphorylation of the remaining hydrophobic phosphopeptides was reduced. Thus it is conceivable that optimum phosphorylation of serine 122 is dependent on the phosphorylation of serine 298. Finally, despite many attempts using alternative strategies, we were unable to replace serines 156 or 158 with alanines probably due to the high GC content in cDNA clones encoding this region of the receptor.

To investigate the importance of phosphorylation in transcription we compared the transcriptional activity of mutants S-122A and S-298A with that of the wild-type receptor in transiently transfected NIH3T3 cells. The wild-type receptor stimulated transcription from the reporter pEREBLCAT by approx. 10-fold with no hormone added and 100-fold (expressed as 100%) in the presence of 10^{-8} M 17β-oestradiol (Fig. 6). Total hormone-dependent transcription was not significantly reduced by either mutation although the basal activity of S-122A in the absence of hormone was reduced to 2–3-fold. We next investigated the effect of combining these serine mutations with mutations in a conserved region near the C-terminus of the receptor found to be important in hormone dependent transcription mediated by AF-2 [20]. Mutation of a number of charged residues (E-546A and D-542N/E-546Q/D-549N) reduced the transcriptional activity of AF-2 alone (data not shown and [20]) but not in the presence of AF-1 (Fig. 6). However, both the basal and the oestrogen dependent transcriptional activity of D-542N/E-546Q/D-549N was reduced by 60–70% when Ser-122 was also mutated. Similarly the transcriptional activity

Table 1. Phosphorylation sites in the mouse oestrogen receptor. After immunoprecipitation radio-labelled receptor proteins was digested with trypsin. The position of phosphopeptides (a–e) was determined by Cerenkov counting and then analysed by solid phase sequencing. Phosphorylated serines are underlined and their position is indicated below in a diagram of the domain structure of the receptor. The receptor is divided into the three domains, the N-terminal activation domain AF-1, the DNA binding domain DBD, and the hormone binding domain that contains another activation domain AF-2

HPLC peak	Peptide number	^{32}P position	Phosphorylation site
a	1, 2, 3	1, 3	156 158 SNSDNR 171 522 LSSSNE or HMSNK
b	5	7	298 WPSPL
c	6, 7, 8	10	122 PQLSPF
d			nd
e	9	10	122 PQLSPF





M L L H P P P Q L S P F L H P H G Q Q Y P Y Y L E
1 10 20

Fig. 3. Micro-sequence analysis of phosphopeptide C. COS-1 cells were labelled with [^{32}P]orthophosphate, or [^{3}H]valine and [^{14}C]tyrosine for 3 h. After immunoprecipitation the receptor was digested with trypsin and peak C was subjected to solid phase sequencing. The positions of [^{32}P]orthophosphate, [^{14}C]tyrosine and [^{3}H]valine were determined by β-scintillation counting and corrected for background radioactivity and ^{32}P carry-over. The position of the phosphorylated serine residue is denoted by an asterisk.

of E-546A, which was approx. 150% that of the wild-type receptor, was reduced to 30% when S-122A/E-546A was analysed and the basal activity was also reduced. Thus the effect of the serine 122 replacement was increased by the introduction of mutations into AF-2 in the hormone binding domain suggesting that the region of AF-1 encompassing serine 122 functionally interacts with AF-2. In addition, it was noteworthy that the replacement of serine 122 also reduced the basal transcriptional activity of the wild-type receptor and both of the mutant receptors.

DISCUSSION

Phosphorylation sites have been identified in the human oestrogen receptor by expressing deletion mutants in COS cells [3, 16] or by direct analysis of the wild-type receptor in MCF-7 mammary carcinoma cells [27, 28] and after over-expression in insect cells [27, 28]. By a process of elimination, sites were mapped in deletion mutants at serines 104 and/or 106 and serine 118 in the N-terminal domain although additional phosphorylation was detected but the sites were not mapped [16]. However, direct sequencing of the major [^{32}P]phosphopeptide derived from the receptor in MCF-7 cells revealed that serine 167 was the major phosphorylation site after oestrogen binding [27].

In our study of the mouse oestrogen receptor we mapped phosphorylation sites directly by solid phase microsequencing of peptides derived from oestrogen receptors that had been radiolabelled *in vivo*. Thus, we detected phosphate incorporation into serines 122, 156, 158, 298 and either Ser-171 or Ser-522. The serine 122 in the mouse receptor is equivalent to serine 118 in the human protein and occurs in a region of the receptor which is highly conserved in other species, including chicken and Xenopus. The Ser-171 corresponds to Ser-167 in the human receptor, reported to be the major phosphorylation site [27], and this too is in a region that is relatively well conserved in other species. Thus, these serines may represent conserved phosphorylation sites that might be involved in common functions. Katzenellenbogen and coworkers [3] found that the replacement of Ser-104 and Ser-106 also reduced phosphorylation of the receptor but the group of Chambon [16] reported that these mutations were

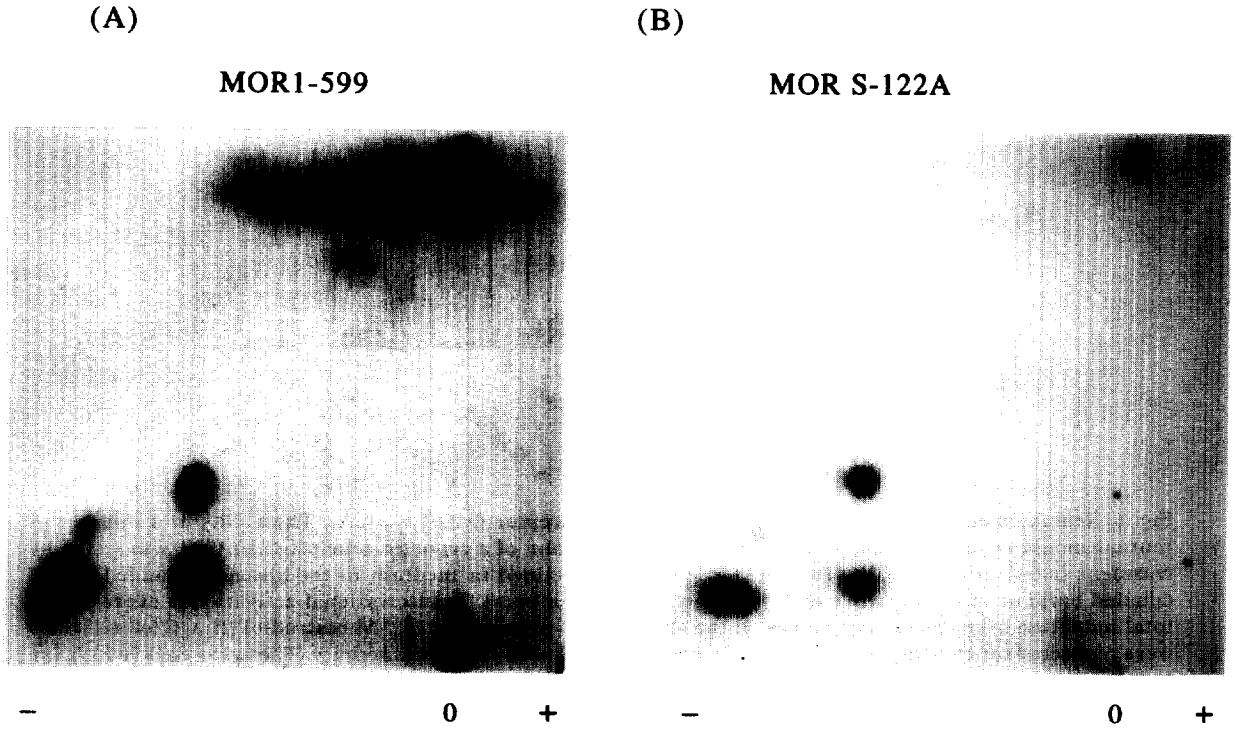


Fig. 4. Two dimensional phosphopeptide map of mutant receptor. Tryptic phosphopeptide maps of the wild-type (A), and mutant MOR S-122A (B) were generated by electrophoresis and chromatography and visualized by autoradiography for 5 days.

without effect. We too were unable to detect a phosphopeptide containing these sites from the mouse receptor although we did not sequence the peptide eluted from the HPLC column in peak (d). On the other hand, we detected phosphorylation on serines 156, 158 and 298 in the mouse receptor and yet mutation of these in the human protein did not result in a change in their phosphopeptide maps. It is noteworthy that the residues surrounding serine 158, or its equivalent in other species, are not conserved and this may account for the difference in phosphorylation between mouse and human. However, the region that includes residue 298 is conserved and so it is difficult to account for species difference in phosphorylation.

The serine, corresponding to residue 118 in the human receptor and 122 in the mouse, appears to play a role in transcriptional activation. Mutation of Ser-118 in the human oestrogen receptor was shown to reduce transcription from reporters in transfected COS-1 and HeLa cells but the effect depended on the promoter

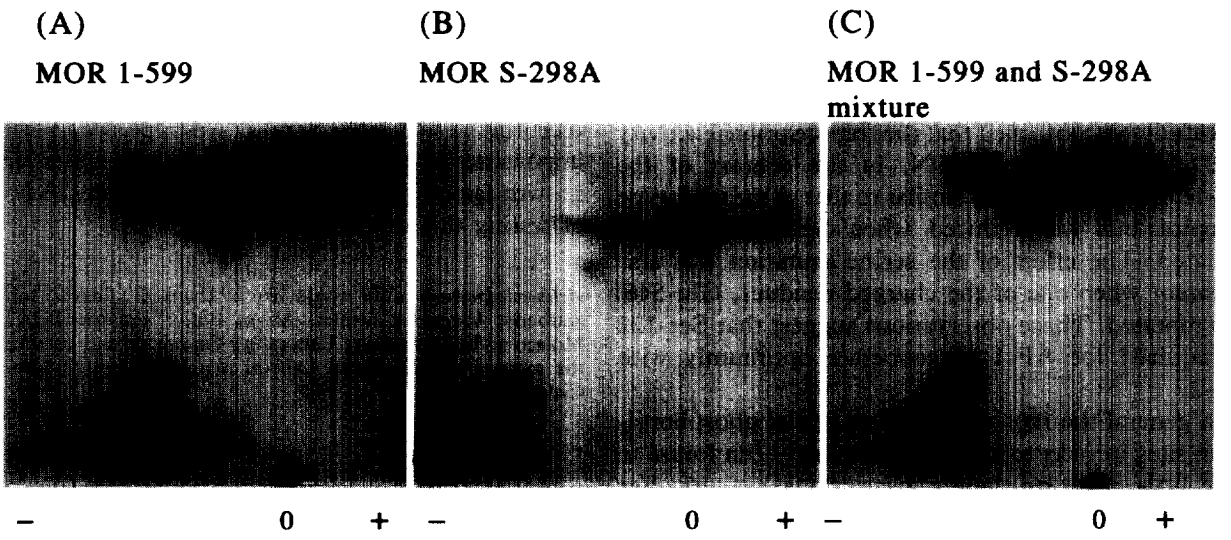


Fig. 5. Two-dimensional phosphopeptide map of mutant receptor. Tryptic phosphopeptide maps of the wild-type (A), and mutant MOR S-298A (B), and the mixture of the two (C) were prepared by electrophoresis and chromatography and visualized by autoradiography for 5 days.

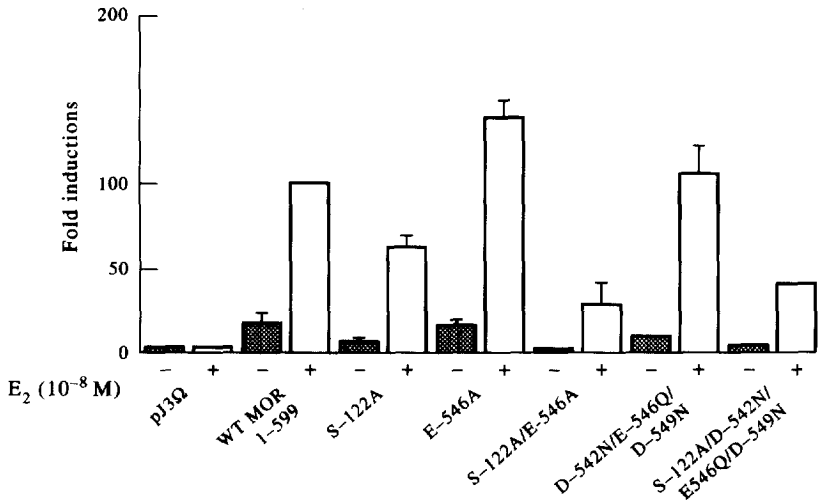


Fig. 6. Transcriptional activation by mutant oestrogen receptor (MOR S-122A). The ability of a number of mutant mouse oestrogen receptors to stimulate transcription of a reporter gene pEREBLCAT was tested by transient transfection in NIH 3T3 cells. Cells were maintained in medium in the absence of added ligand (shaded bars) or in the presence of 10^{-8} M oestradiol (open bars). Transcriptional activation is expressed as total induction over the reporter alone in the absence and presence of 10^{-8} M oestradiol (E_2). CAT activities were corrected for the differences in transfection efficiency with luciferase activity. The error bars (which are too small to be visible in some cases) represent standard errors determined from at least three transfection experiments, each carried out in duplicate. The expression vector alone is shown as pJ3Ω.

and cell-type [16]. Transcriptional activation was unaffected in chicken embryo fibroblasts, which is surprising given that the activity of AF-1 is higher in these cells than most other cells [29, 30]. When Ser-122 in the mouse receptor was replaced by alanine it retained its ability to stimulate transcription of the reporter EREBLCAT in NIH3T3 cells albeit with a reduction of 30–40%. However a number of different approaches have been used to demonstrate that AF-1 and AF-2 cooperate with one another [20, 31, 32] and so we investigated the effect of the mutation on this co-operation. We have previously demonstrated the importance of a putative amphipathic α-helix for hormone dependent transcription [20] and showed that while transcription by the hormone binding domain was abolished by mutation of the charged residues it could be rescued by the presence of AF-1, consistent with the notion of cooperativity between AF-1 and AF-2. In this study we confirmed that the transcriptional activity of D-542N/E-546Q/D-549N, in the context of the full-length receptor, was similar to that of the wild-type receptor but was reduced when Ser-122 was also mutated. The effect of the serine mutation was also apparent when one of the charged residues, Glu-546, was mutated. These observations suggest that Ser-122 is required for AF-1 to cooperate optimumly with AF-2.

In general the replacement of potential phosphorylation sites in receptors with alanine had been found to have little effect on their transcriptional activity. For example the transcriptional activity of the glucocorticoid receptor was retained even when all of the phosphoserines that had been previously identified were mutated [33]. Therefore, providing transient transfection assays are a reliable measure of the transcriptional activity of the receptor, it is conceivable that phosphorylation is important for another function, such as processing and shuttling [1, 11] or DNA binding [12], which for some reason is not apparent simply by measuring transcriptional activity. Alternatively, phosphorylation might alter the sensitivity of the receptor to other signalling pathways such as those mediated by protein kinase A [34, 35]. Another possibility that we are investigating is that Ser-122 represents a potential target for extracellular signal-regulated kinases ERK1/ERK2 that are activated by growth factors by means of the MAP kinase pathway. The growth factor EGF has been shown to stimulate the transcriptional activity of the oestrogen receptor and the N-terminal activation domain AF-1 was found to be essential for the effect [36]. It is conceivable that the reduction in the basal transcriptional activity observed with mutant receptors in which the Ser-122 was replaced reflects the importance of this residue in mediating the effects of growth factors present in serum. We are in the process of testing this directly.

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