

Application of phosphorylation site-specific antibodies to measure nuclear receptor signaling: characterization of novel phosphoantibodies for estrogen receptor α

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An understanding of posttranslational events in nuclear receptor signaling is crucial for drug design and clinical therapeutic strategies. Phosphorylation is a well-characterized posttranslational modification that regulates subcellular localization and function of nuclear receptors and coregulators. Although the role of single phosphorylation sites in nuclear receptor function has been described, the contribution of combinations of multiple phosphorylation sites to receptor function remains unclear. The development of phosphoantibodies to each phosphorylation site in a nuclear receptor is a powerful tool to address the role of phosphorylation in multiply phosphorylated receptors. However, phosphoantibodies must be rigorously validated prior to use. This review describes the general methodology for design, characterization and validation of phosphoantibodies using the example of eight phosphoantibodies raised against phosphorylation sites in estrogen receptor α (ER α).

Received November 10th, 2005; Accepted February 21st, 2006; Published April 28th, 2006 | **Abbreviations:** A: Alanine; E2: 17 β -estradiol; ER α : Estrogen receptor α ; PKA: Protein Kinase A; S: Serine; T: Threonine; Y: Tyrosine | Copyright © 2006, Al-Dhaheeri and Rowan. This is an open-access article distributed under the terms of the Creative Commons Non-Commercial Attribution License, which permits unrestricted non-commercial use distribution and reproduction in any medium, provided the original work is properly cited.

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Introduction

Phosphorylation is the well-established posttranslational event that regulates subcellular localization, dimerization, DNA binding, coregulator interaction and transcriptional activity of nuclear receptors [Orti et al., 1992]. Understanding the role of phosphorylation in nuclear receptor function is limited by receptor expression levels, intracellular phosphatase activity, and low stoichiometry and/or rapid turnover of some phosphorylation sites.

Several approaches may be used to study previously identified phosphorylation sites in nuclear receptors [Rowan et al., 2003]. Cells may be labeled *in vivo* with ^{32}P orthophosphate followed by immunopurification and phosphopeptide mapping. Similarly, purified receptor may be phosphorylated *in vitro* with different kinases and individual sites assessed by phosphopeptide mapping [Rowan et al., 2003]. However these approaches are labor intensive, time consuming, and require radioactive material and large amounts of purified receptor. Mass spectrometry is another approach to study protein phosphorylation [Garcia et al., 2005]. This non-quantitative approach requires large amounts of purified receptor and is limited primarily by costly instrumentation and the need for a highly skilled operator.

The phosphoantibody approach to study receptor phosphorylation overcomes several limitations inherent in other approaches and provides the additional benefit of allowing rapid assessment of combinations of receptor phosphorylation sites by Western blotting [Rowan et al., 2003]. The advantages of the phosphoantibody approach

include: 1) a highly sensitive assay that can detect phosphorylation in crude extracts containing low receptor levels and/or low stoichiometry of phosphorylation without the need for purified receptor; 2) the ability to profile total receptor phosphorylation and to quantify relative levels of each phosphorylation site; 3) immunoprecipitation of receptors phosphorylated at specific sites to investigate the recruitment of phosphoproteins to promoters (chromatin immunoprecipitation (ChIP)) and to identify phosphorylation sites involved in specific protein-protein interaction (co-immunoprecipitation); 4) identification of phosphorylation sites associated with real time dynamics of receptor subcellular localization and recycling and other cellular processes (immunofluorescence; flow cytometry sorting); 5) the ability to monitor changes in the total receptor phosphorylation profile during disease processes such as the progression from benign to malignant cancer (immunohistochemistry).

The phosphoantibody approach has been widely applied to characterize nuclear receptor activity and subcellular localization [Wang et al., 2002]. Glucocorticoid receptor (GR) GR-P-S203 and GR-P-S211 phosphoantibodies have been used by immunoblotting to study GR activation by different ligands and by indirect immunofluorescence to investigate the subcellular localization of the active GR. Progesterone receptor (PR) phosphospecific antibodies to sites S400 [Pierson-Mullany and Lange, 2004], S162, S190 and S294 [Narayanan et al., 2005] were used by immunoblotting to study the ligand independent activation of PR and to correlate PR transcriptional activity and cell-cycle progression.

Androgen receptor (AR) phosphoantibody to site S81 was used by immunoblotting to study the mechanism of ligand-dependent arrest of the AR in subnuclear foci [Black et al., 2004]. Phosphoantibodies to ER α sites S118 and S167 [Chen et al., 2002; Shah and Rowan, 2005] were used by immunoblotting to study crosstalk between ER α and several kinases. In addition, these phosphoantibodies have been used by immunohistochemistry to detect ER α phosphorylation in human breast tumors [Murphy et al., 2004; Yamashita et al., 2005].

The following section describes six key steps in development and characterization of phosphoantibodies to study nuclear receptor phosphorylation (Figure 1). The first three steps include preparation, purification and initial testing of phosphoantibodies and are generally performed by commercial vendors. The remaining three steps to validate and confirm specificity of the phosphoantibody for specific phosphorylation sites are performed by the investigator in the laboratory and are the major focus of this review.

Peptide design and immunogen preparation

The synthetic phosphopeptide used for immunization is recommended to be 10-20 amino acids in length and conjugated with a suitable carrier that will solicit a strong immune response and produce a large quantity of the antibody [Eisele et al., 1999; Frank, 1984; Harlow and Lane, 1988]. The most widely used carriers in antibody production are keyhole limpet hemacyanin (KLH) and bovine serum albumin (BSA) [Harlow and Lane, 1988].

It is important to be aware of the conjugated carrier used during immunization to anticipate any false positives that may occur during antibody testing. For example if the conjugated carrier is BSA, a false positive may arise if BSA was used as a blocking agent for Western blotting. Immunogenicity of the peptide-protein carrier can be verified by an enzyme linked immunosorbent assay (ELISA) to identify the most appropriate protein carrier dose.

Producing hyper-immune serum

For broad specificity it is recommended that phosphoantibodies be prepared as polyclonal antibodies [Burns, 2005; Harlow and Lane, 1988; Leenaars and Hendriksen, 2005; Lipman et al., 2005]. Rabbits are the host of choice to avoid self-recognition of the immunogen and since rabbits provide high amounts of sera. The immunogen is mixed with adjuvant prior to immunizing animals.

The immunogen mixture is injected subcutaneously and re-administered at day 14 and 44 post immunization. At day 54 sera is collected by bleeding of the marginal ear vein. Bleeding is repeated on day 60 and then every 4 weeks until the antibody titer has declined. A booster dose of immunogen should be administered to re-enhance animal immunity and produce more sera [Burns, 2005]. After each bleeding, antibody titer should be measured by ELISA.

Affinity Purification

Following harvest of the hyper-immune sera, the phosphoantibody can be enriched by an affinity purification against the phosphorylated peptide [Harlow and Lane, 1988]. The affinity purification is validated by ELISA screening against the phosphopeptide in which phosphoantibody purity should exceed 95%. Numerous commercial suppliers will produce custom, affinity purified phosphoantibodies (Bethyl Laboratories, Invitrogen, Global Peptide, ABGENT, Open Biosystems and others).

Phosphoantibody specificity assessed by receptor phosphorylation site mutations

Affinity purified phosphoantibody must be validated as phosphorylation site specific. In mammalian cells that do not express the receptor of interest, wild type receptor expression plasmids or plasmids containing phosphorylation site mutations (Serine (S) to Alanine (A), Threonine (T) to A, Tyrosine (Y) to Phenylalanine (F)) should be transfected in cells to achieve high level protein expression. Total cell extracts from wild-type and phosphorylation site mutant transfected cells should be prepared for Western blotting with the phosphoantibody and peptide-competed phosphoantibody to assess specificity for the phosphorylation site. High receptor levels may be required to measure phosphorylation sites that exhibit low stoichiometry. For other sites that require an activation event, cells may first need to be incubated with ligand, growth factor, kinase activator, etc. prior to detection of the specific phosphorylation with a phosphoantibody.

The ideal phosphoantibody should recognize receptor from cells transfected with wild type but not the site-specific mutant protein. However depending on cell context, protein expression and stoichiometry, some phosphoantibodies may fail to detect a signal in total cellular extract. If this occurs, enrichment of receptor by immunoprecipitation with a receptor-specific antibody is recommended prior to Western blotting with the phosphoantibody. It is critical that immunoprecipitation be carried out over a brief period (no more than a few hours) since dephosphorylation may occur over longer periods. Regardless, phosphatase inhibitors should always be included in lysis buffers whether receptor is immunoprecipitated or not.

A critical step when first using phosphoantibodies for Western blotting is to empirically determine the optimal phosphoantibody concentration that will: 1) exhibit reactivity with wild type, but not phosphorylation site mutant receptor; and 2) not result in high general background on Western blots. In our experience, some phosphoantibodies exhibit specificity for wild type and not phosphorylation site mutant receptor only at high antibody dilutions. The appropriate antibody dilution must be determined empirically using serial dilutions of the affinity purified antibody in Western blot analysis. We have found that some affinity purified phosphoantibodies (stock concentration of 1 mg/ml) require dilution as high

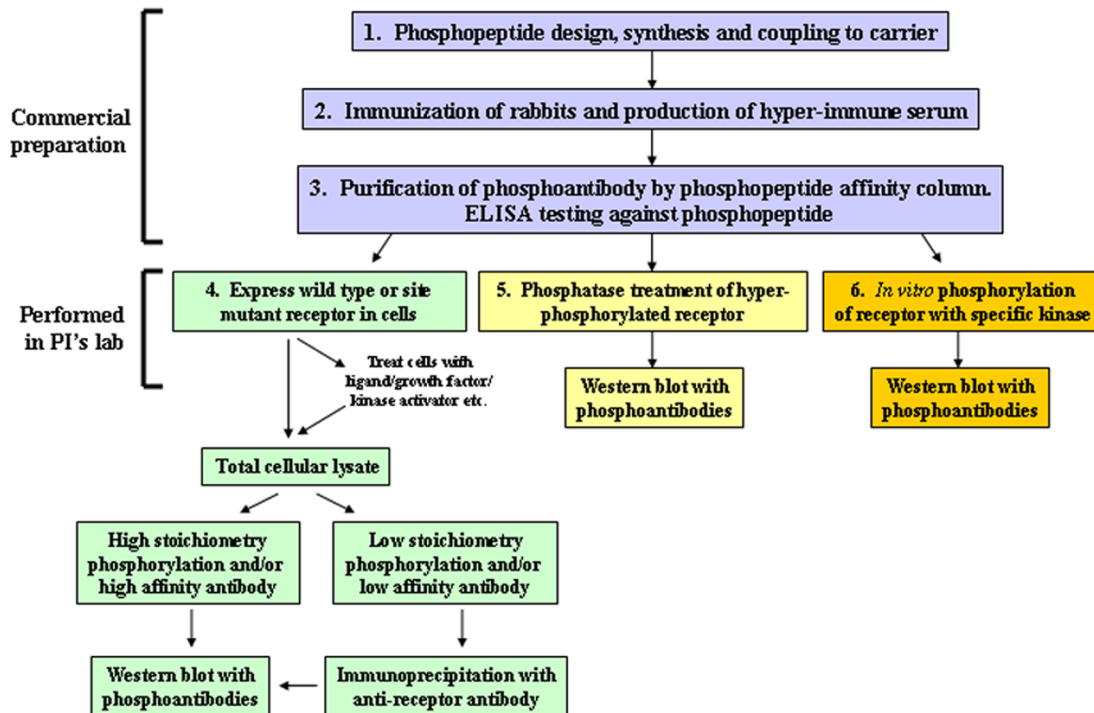


Figure 1. Steps in phosphoantibody production and validation. Phosphopeptide design, rabbit immunization, phosphoantibody production and affinity purification (steps 1-3) are performed by commercial vendors. Phosphoantibody specificity experiments (steps 4-6) are performed by the investigator.

as 1:10,000 to eliminate non-specific reactivity with the phosphorylation site mutant receptor.

Absence of phosphoantibody reactivity with de-phosphorylated receptor

A second step for validation of phosphoantibodies is to confirm the antibody does not react with receptor that has been de-phosphorylated. In this context, the hyper-phosphorylated form of the purified receptor should be incubated in the presence or absence of λ phosphatase, followed by Western blotting with the phosphoantibody. Phosphatase treatment should prevent reactivity of the phosphoantibody with the receptor.

in vitro receptor phosphorylation to measure phosphoantibody reactivity

In some cell/tissue contexts that lack a specific kinase or have inactive kinase, some phosphoantibodies may not react with receptor due to absence or very low stoichiometry of phosphorylation at a particular site. In this scenario, *in vitro* phosphorylation of purified receptor with a kinase known to phosphorylate the specific site can be used prior to incubation with λ phosphatase. Phosphoantibody reactivity with the purified protein should increase following *in vitro* phosphorylation and signal should be lost following λ phosphatase treatment. A kinase that is not specific for the site in question should be used as a control for the *in vitro* phosphorylation.

Reactivity and specificity: phosphoantibodies to ER α as an example

In this study, phosphoantibodies developed against eight different phosphorylation sites of ER α were characterized (Figure 2). ER α is phosphorylated upon ligand binding and/or crosstalk with kinases. Although there are eight identified phosphorylation sites in ER α , (S104, S106, S118, S167, S236, S305, T311, and Y537 [Lannigan, 2003; Michalides et al., 2004], only phosphoantibodies against S118 and S167 have been widely applied for receptor functional studies. Phosphoantibodies directed against S104, S106 and Y537 are also commercially available although application of these antibodies to receptor functional studies is limited. This may be due to lower stoichiometry of phosphorylation at these sites, rapid turnover of tyrosine phosphorylation sites and/or poorer affinity of these antibodies compared to the S118 and S167 phosphoantibodies.

Reagents

Antibodies

Bethyl Laboratories (Montgomery, TX) provided complimentary phosphoantibodies: ER α ER-P-S104 (Cat. # BL1637), ER-P-S106 (Cat. # BL1638), ER-P-S118 (Cat. # BL1641), ER-P-S167 (Cat. # BL1643), ER-P-S236 (Cat. # 1645), ER-P-S305 (Cat. # 1665), ER-P-T311 (Cat. # BL1667) and ER-P-Y537 (Cat. # BL1647). Antibody D12 for ER α immunoprecipitation (Cat. # sc-8005) was purchased from Santa Cruz Biotechnology (Santa Cruz,

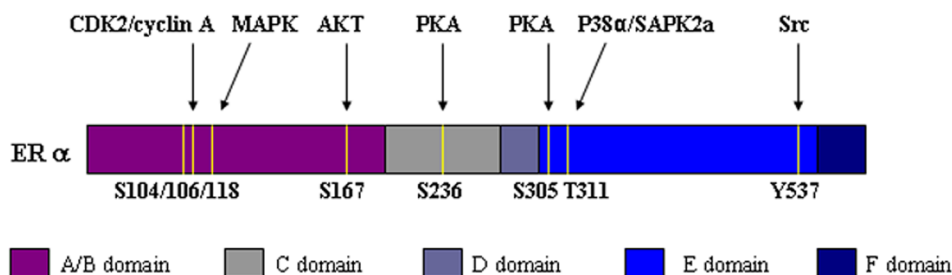


Figure 2. Schematic representation of ER α phosphorylation sites. ER α phosphorylation sites that have been confirmed in previous studies by *in vivo* and/or *in vitro* phosphorylation are shown.

CA). ER α antibody clone 6F11 for Western blots of total ER α (Cat. # VP-E613), peroxidase conjugated anti-mouse (Cat. # π -2000) and peroxidase conjugated anti-rabbit antibodies (Cat. # π -1000) were purchased from Vector Laboratories (Burlingame, CA).

Purified ER α , phosphatase and kinases

Baculoviral expressed ER α (Cat. # P2187) was purchased from Invitrogen (Carlsbad, CA). λ protein phosphatase (Cat. # P0753S) and active recombinant full length human CDK2-cyclin A complex expressed in *E. coli* (Cat. # P6025S) were purchased from New England Biolabs (Ipswich, MA). Baculoviral expressed, active recombinant full length human protein kinase A (PKA) catalytic subunit β (Cat. # PPK-448) was purchased from Stressgen (San Diego, CA). Baculoviral expressed, active recombinant full length human Src kinase (Cat. # 14-326) and active recombinant human full length p38 α /SAPK2a kinase expressed in *E. coli* (Cat. # 14-587) were purchased from Upstate (Charlottesville, VA).

Methods

Cell culture and transfection

COS-1 cells were maintained in DMEM phenol red free medium containing 10% Fetal Bovine Serum (FBS), 2% glutamine and 1% penicillin/streptomycin. Cells were plated in 15 cm dishes for 48 hrs in DMEM phenol red-free medium containing 5% FBS that was treated with dextran-coated charcoal to remove endogenous steroids. Wild type or phosphorylation site mutant ER α constructs (6 μ g) were transiently transfected in cells for 48 hrs using Fugene 6 (Roche, Indianapolis, IN). Cells were incubated with 17 β -estradiol (E2) (10 nM) or Forskolin (10 μ M) + IBMX (100 μ M) (F/I) for 2 hrs. Cells were harvested and cellular pellets were incubated in high salt lysis buffer (10 mM Tris pH 8.0, 0.4 M NaCl, 2 mM EDTA pH 8.0, 2 mM EGTA, 10 mM β -mercaptoethanol, 0.1% Triton X100, 1 mM sodium orthovanadate, 20 mM β -glycerophosphate, 25 mM sodium fluoride, 0.1 mM PMSF) containing protease inhibitor mixture (σ , St. Louis, MO) for 10 min in ice.

Immunoprecipitation of ER α

Protein A sepharose beads (Amersham Biosciences, Piscataway, NJ) were re-swelled in phosphate buffer saline (PBS) and incubated with ER α antibody (D12, Santa Cruz) by rotating the beads at room temperature for 2 hrs. Beads were washed three times with PBS, followed by incubation with the total COS-1 cellular extract for 3 hrs at 4 C with rotation. Beads were washed three times with PBS, followed by addition of 5X SDS-PAGE loading buffer. Beads were incubated at 100 C for 5 min to elute ER α . Samples were electrophoresed by SDS-PAGE and phosphorylation of ER α was detected by Western blotting using site specific phosphoantibodies. Phosphoantibody signals obtained by Kodak Image analysis of Western blots were normalized to total ER α level by incubating the membrane in stripping buffer (2% SDS, 100 mM β -mercaptoethanol, 62.5 mM Tris HCl pH 6.8) at 55 C for 30 min with rotation and re-probing the membrane by Western blot with antibody against total ER α (clone 6F11, Vector Laboratories).

λ Phosphatase analysis

200 ng purified ER α was incubated with 200ng λ phosphatase in reaction buffer (50mM Tris-HCl, 100 mM NaCl, 2 mM MnCl₂ 2mM dithiothreitol (DTT), 0.1 mM EGTA, 0.01 % Brij 35, pH 7.5) for 1 hr at 30 C. To terminate the reaction, 5X SDS-PAGE loading buffer was added and samples were incubated at 100 C for 5 min., followed by SDS-PAGE and Western blots using site specific phosphoantibodies

in vitro phosphorylation

Purified ER α (200 ng) was incubated with the site specific kinase or kinase complex, ATP, and kinase buffer for 1 hr at 30 C in the presence or absence of λ phosphatase. Kinases used were: CDK2-cyclin A (100 ng; (kinase buffer 50 mM Tris-HCl, 10 mM MgCl₂, 1 mM EGTA, 2 mM DTT, 0.01% Brij 35, pH 7.5); PKA (100 ng) or inactive PKA (PKA incubated at 100 C for 2 min.; kinase buffer (20 mM MOPS pH 7.0, 1 mM sodium orthovanadate, 25 mM β -glycerophosphate, 1 mM EGTA, 1 mM DTT, 7 mM MgCl₂); P38 α /SAPK2a (100 ng; (kinase buffer 25 mM

Tris-HCl pH 7.5, 0.02 mM EGTA); and Src kinase (100 ng; kinase buffer 8 mM MOPS pH 7.0, 0.2 mM EDTA). *in vitro* phosphorylation reactions were terminated by addition of 5X SDS-PAGE loading buffer and incubation of samples at 100 C for 5 min. Following SDS-PAGE, phosphorylation of S104, S236, S305, T311, and Y537 were detected by Western blotting with phosphoantibodies.

Results

The initial step for characterization of phosphoantibodies is to prove the antibody is site specific. For this purpose, wild type ER α and site mutant ER α constructs were separately transfected into ER α -negative COS-1 cells. Cells were then incubated with 17 β -estradiol (10-8M) or forskolin (10 μ M) + IBMX (100 μ M) for 2 hrs and cell extracts were prepared for Western blotting. Phosphorylation sites with low stoichiometry or antibodies with low affinity may not detect receptor in crude cellular extracts. For this reason ER α was first immunoprecipitated with a separate ER α antibody (D12, Santa Cruz) and then the immunopurified receptor was subjected to Western blotting with the phosphoantibodies. Phosphoantibodies to ER α -P-S104 (Figure 3A), ER α -P-S106 (Figure 3B), ER α -P-S118 (Figure 3C), ER α -P-S167 (Figure 3D), ER α -P-S236 (Figure 3E), ER α -P-S305 (Figure 3F), ER α -P-T311 (Figure 3G) and ER α -P-Y537 (Figure 3H) detected wild type ER α , but not phosphorylation site ER α mutants, indicating that antibodies were site specific. For some phosphoantibodies serial dilutions were prepared to determine the optimal antibody concentration for detection of wild type, but not mutant ER α . At high concentration (2 μ g/ml) the ER α -P-S167 phosphoantibody recognized both wild-type ER α and mutant S167A (data not shown). This nonspecific interaction with mutant S167A was absent when the antibody concentration was decreased to 0.25 μ g/ml (Figure 3D).

Specificity of the antibodies was verified by λ phosphatase treatment of the hyper-phosphorylated purified ER α or by *in vitro* phosphorylation of ER α with specific kinases. Phosphoantibodies to sites S106, S118 and S167 recognized purified, baculoviral expressed ER α , indicating that kinase pathways that phosphorylate these sites are conserved in insect Sf9 cells (Figure 4 A-C, lane 1). A similar conservation between mammalian and Sf9 insect cells for receptor phosphorylation was observed with baculoviral expressed progesterone receptor [Beck et al., 1996]. Purified ER α was incubated with λ phosphatase for 1 hr at 30 C and the phosphorylation of S106, S118, and S167 was assessed by Western blotting with the phosphospecific antibodies. λ phosphatase treatment of ER α resulted in loss of Western blot signal with phosphoantibodies to sites S106 (Figure 4A), S118 (Figure 4B) and S167 (Figure 4C).

Unlike phosphoantibodies to S106, S118, and S167, phosphoantibodies to sites S104, S236, S305, T311 and Y537 exhibited no reactivity with purified ER α (data not shown). The antibody specificity was assessed by *in vitro* phosphorylation of ER α with CDK2-cyclin A, PKA,

p38 α /SAPK2a and Src kinases followed by Western blotting with phosphoantibodies to sites S236, S305, T311 and Y537.

CDK2-cyclin A significantly induced the phosphorylation of site S104 (Figure 5A, lane 2) and subsequent λ phosphatase treatment (Figure 5A, lane 3) abolished the phosphorylation as assessed by Western blotting with the ER α -P-S104 phosphoantibody. In the absence of kinase, incubation of purified baculoviral ER α with kinase buffer and ATP also resulted in phosphorylation of site S104 (Figure 5A, lane 1), suggesting that purified, baculoviral ER α may be contaminated with a co-purifying kinase.

Incubation of ER α with active PKA, but not inactive PKA, resulted in phosphorylation of ER α at S236 (Figure 5B, lane 2 versus lane 1) and S305 (Figure 5C, lane 2 versus lane 1). *in vitro* phosphorylation of ER α with P38 α /SAPK2a complex resulted in T311 phosphorylation (Figure 5D, lane 3), and this phosphorylation was lost with subsequent λ phosphatase incubation (Figure 5D, lane 2). Incubation of ER α with Src kinase resulted in phosphorylation of ER α at Y537 (Figure 5E, lane 2) and this phosphorylation was markedly reduced with subsequent λ phosphatase incubation (Figure 5E, lane 3).

Discussion

Although there are several different methods to characterize changes in receptor phosphorylation, the phosphoantibody approach is particularly useful for simultaneously measuring each phosphorylation site. In this report the general steps in characterizing and validating phosphoantibodies are described using the example of phosphoantibodies raised against eight phosphorylation sites in ER α . Validation of each phosphoantibody was assessed in two steps; first the site specificity of the phosphoantibody was validated by expressing ER α wild type or site mutant plasmids into receptor negative cell lines. Second, the phosphoantibody specificity was validated by incubating the hyper-phosphorylated purified ER α with λ phosphatase and by performing *in vitro* phosphorylation with a specific kinase or kinase complex that is known to phosphorylate a specific site.

Although all eight phosphoantibodies raised against each ER α phosphorylation site were site specific, these phosphoantibodies reacted differently with the hyper-phosphorylated, baculoviral expressed ER α . Only phosphoantibodies to sites S106, S118 and S167 recognized the baculoviral expressed ER α . This may have occurred because some sites are not phosphorylated in insect Sf9 cells due to either the absence of kinase activity or the presence of phosphatases that prevent ER α phosphorylation at these sites.

CDK2-cyclin A is a specific kinase complex previously shown to phosphorylate both S104 and S106 of ER α [Rogatsky et al., 1999]. Because of the close proximity

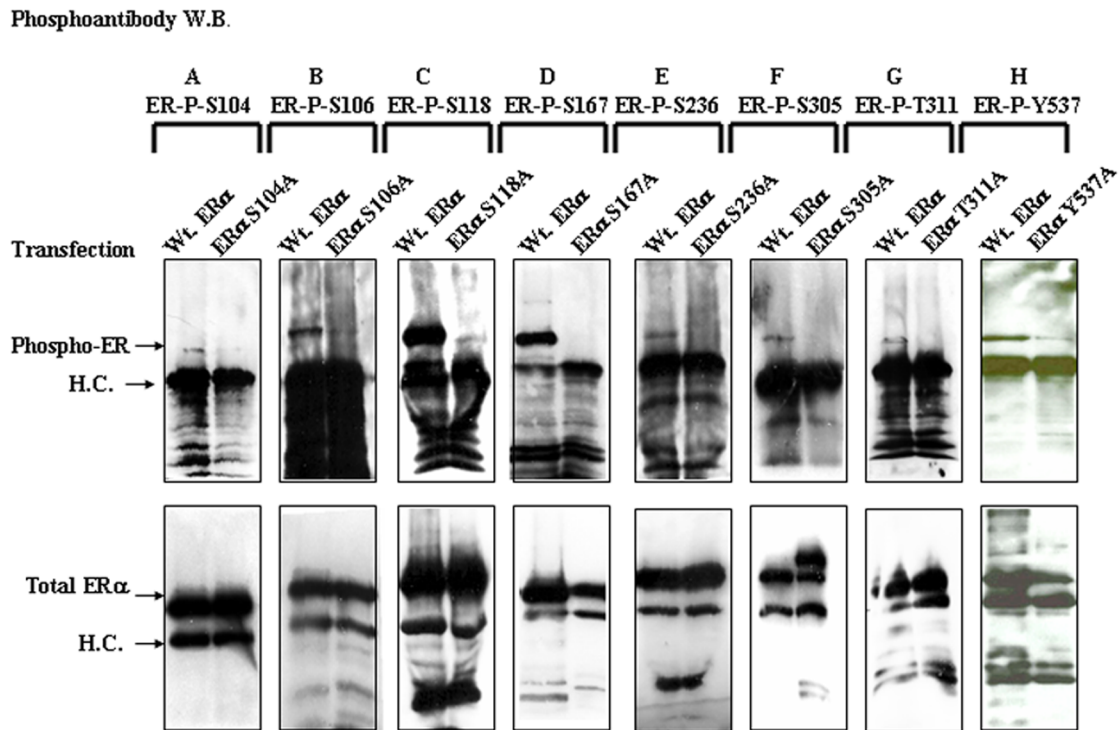


Figure 3. Immunoprecipitation of wild type and mutant ER α and Western blotting with phosphoantibodies. COS-1 cells were plated in DMEM media containing 5% FBS treated with charcoal coated dextran to remove the endogenous steroids. Cells were transfected with wild type ER α (A-H) or ER α phosphorylation site mutants ER α S104A (A), ER α S106A (B), ER α S118A (C), ER α S167A (D), ER α S236A (E), ER α S305A (F), ER α T311A (G), or ER α Y537A (H). Cells were incubated with 17 β -estradiol (A-D, G, H) or F/I (E, F) for 2 hrs, followed by preparation of cell extracts for immunoprecipitation. Total ER α was immunoprecipitated with ER α antibody (D12, Santa Cruz) and phosphorylation of ER α was detected by Western blot using site specific phosphoantibodies. The immunoblot was stripped and re-probed by Western blot with antibody to total ER α (Clone 6F11, Vector Laboratories).

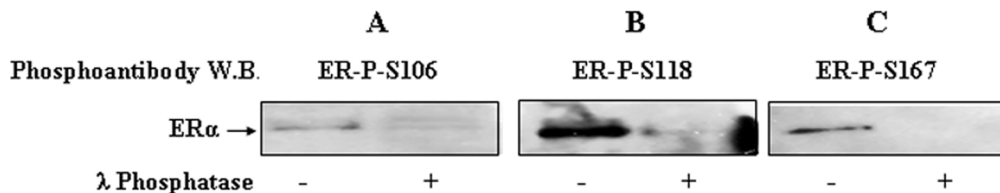


Figure 4. Phosphatase treatment of purified ER α and Western blotting with phosphoantibodies. Purified ER α (200ng) was incubated in the presence or absence of phosphatase λ (200ng) at 30 °C for 1 hr and phosphorylation of ER α was detected by Western blotting using ER α -P-S106 (A), ER α -P-S118 (B), or ER α -P-S167 (C) phosphoantibodies.

of S104 and S106, it is possible that phosphorylation of one site may be required for phosphorylation of the adjacent site (currently under investigation). Serine 236 and Serine 305 are located within consensus sequences for PKA and phosphorylation of these sites by PKA has been demonstrated [Chen et al., 1999; Michalides et al., 2004]. The direct kinase that phosphorylates ER α at T311 has not been identified. Lee and Bai [Lee and Bai, 2002] reported that T311 is phosphorylated by a p38 MAPK

kinase complex that contained both p38 α and SAPK2a. Since T311 does not lie within a consensus sequence for MAPK phosphorylation (the T residue is not followed by a proline residue), it is highly unlikely that p38 directly phosphorylates T311. A more likely explanation is that the kinase that directly phosphorylates T311 is a kinase that copurified with p38 in the present and previous study. Y537 is present within a Src kinase consensus sequence

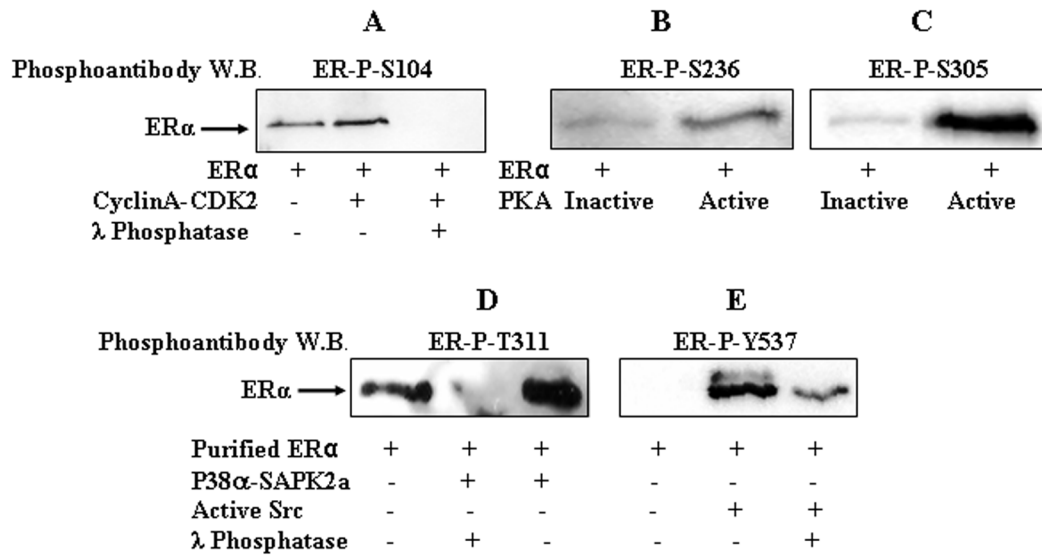


Figure 5. *in vitro* phosphorylation of purified ER α and Western blotting with phosphoantibodies. Purified ER α (200 ng) was incubated with or without CDK2-cyclin A complex (100 ng) (A), PKA (100 ng) (B, C), P38 α /SAPK2a complex (100 ng) (D), or Src (100 ng) (E) in the presence or absence of λ phosphatase (200 ng) (A, D, E) for 1 hr at 30°C. PKA was inactivated by incubating the kinase at 100°C for 2 min. ER α phosphorylation was detected by Western blot analysis using ER α -P-S104 (A), ER α -P-S236 (B), ER α -P-S305 (C), ER α -P-T311 (D), or ER α -P-Y537 (E) phosphoantibodies.

and is phosphorylated by Src kinase *in vitro* [Arnold and Notides, 1995; Arnold et al., 1995].

In summary, development of phosphoantibodies to assess the functional role of nuclear receptor phosphorylation is a powerful approach that has distinct advantages over other more labor intensive and costly approaches. However, rigorous validation experiments must precede use of phosphoantibodies to ascertain both reactivity with the phosphorylated receptor and absence of cross reactivity with non-phosphorylated receptor. Phosphoantibody specificity is determined using several complimentary approaches, including expression of wild type and mutant receptor in cells, phosphatase treatment of receptor, and *in vitro* phosphorylation of receptor. Using phosphoantibodies that are specific for each identified phosphorylation site will permit investigators to simultaneously measure the entire profile of receptor phosphorylation during studies that probe nuclear receptor mechanisms of action.

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