Hormonal Regulation and Identification of Chicken Progesterone Receptor Phosphorylation Sites*

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The present studies examine the effects of in vivo and in situ progesterone treatment in the regulation of site-specific phosphorylation of the chicken oviduct progesterone receptor (PR). By gas-phase protein sequencing we have identified three hormonally regulated phosphorylation sites: Ser-211, Ser-260, and Ser-530. We determined phosphorylation stoichiometries by analyzing the amounts of phosphorylated and dephosphorylated serine at each site. Stoichiometries of sites 211 and 260 were about 20% under basal conditions and increased 1.5–2-fold by in situ progesterone treatment. Site 530 was virtually absent under basal conditions and induced to >33% by in situ progesterone treatment. We tested several protein kinases for phosphorylation of the PR in vitro on these sites or peptides containing these sites. We found that the catalytic subunit of cAMP-dependent protein kinase mimicked the in vivo, hormone-induced altered mobility of the PR in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Both the in vivo and in vitro alterations were reversed by alkaline phosphatase. Finally, we showed that cAMP-dependent protein kinase phosphorylated Ser-528.

The steroid hormone receptors are members of a superfamily of ligand-stimulated transcription factors (1). Although the receptors for progesterone (2–7), estrogen (8), glucocorticoids (9–14), vitamin D (15), and thyroid hormone (16, 17) are phosphorylated in situ, the role of site-specific phosphorylation in the regulation of specific receptor functions remains unclear. Receptor-mediated transcription, DNA binding (19), and hormone binding (20–22) have all been proposed as receptor functions regulated by phosphorylation. All steroid receptors studied are phosphorylated on multiple sites, but the precise location of these sites within the molecules is unknown. Indirect localization experiments using antibody epitope mapping and partial proteolytic or chemical cleavage have been inconsistent regarding the position of phosphorylation sites. For example, it has been suggested that all progesterone receptor (PR) sites are on the amino-terminal side of the hormone (23) or DNA (24) binding domains. Glucocorticoid receptor sites have been suggested to be in only the amino-terminal (25), or both the amino- and carboxyl-terminal (26) portions of the receptor. Due to the existence of multiple phosphorylation sites, it has been difficult to define functional or structural roles for these modifications.

In the present study we have identified three phosphorylation sites by protein sequencing and determined their stoichiometry and hormonal regulation in the chicken oviduct PR. This receptor consists of two ligand-binding forms, PRα (Mr = 72,000) and PRβ (Mr = 86,000), which arise by alternate initiation of translation from the same primary transcript (27). Based on the sequence of PRα, two hormone-stimulated sites (Ser-211 and Ser-260) are in the amino-terminal domain while one hormone-induced site (Ser-530) is in one of the PR transactivation domains in the hinge region between the DNA and hormone binding domains. All three hormonally regulated PR sites contain consensus sequences for the proline-directed kinase (28) and conform to the Ser/Thr-Pro motif (serine or threonine followed by proline) postulated to be important for functional regulation of transcription factor activity (29). Additionally, Ser-260 is part of a consensus sequence for cAMP-dependent protein kinase (PKA).

In the present work we also studied kinases and phosphatases that regulate PR phosphorylation. We show that in vitro phosphorylation of the chick PR with PKA mimics the in vivo, hormone-dependent decreased mobility of receptors in SDS-PAGE seen for chick (5, 19, 30), human (4), and rabbit (3) PRs. The PKA-induced altered mobility can be reversed by alkaline phosphatase in vitro. We also show that the in vivo-induced altered mobility is reversed in vitro with alkaline phosphatase, similar to the human PR (31). Finally, by direct protein sequence analysis we show that PKA phosphorylates Ser-528 in vitro, a serine adjacent to the hormonally induced Ser-530. The potential roles of these sites are discussed in the context of our observations that PKA regulates PR-mediated transcription.

MATERIALS AND METHODS

Materials—Carrier-free [32P]HPO4 (385 Ci/mg of phosphorus) and [γ-32P]ATP were from ICN. 1-Thioglycerol and 2-mercaptoethanol were from Sigma. The monoclonal anti-chicken PR antibody (PR22) was kindly provided by Dr. David O’Toff (Mayo Clinic, Rochester, MN) (32). Rabbit anti-mouse IgG was from Zymed (South San Francisco, CA). Protein A-Sepharose was from Pharmacia LKB Biotechnology Inc. Acrylamide was from Serva Fine Biochemicals Inc., Garden City Park, NY. All other gel electrophoresis supplies were from Bio-Rad. Tosylphenylalanlyl chloromethyl ketone-treated trypsin was from CooperBiomedical, Inc., Malvern, PA. Sequencing grade endopeptidases Asp-N and Glu-C, and molecular biology grade calf intestinal alkaline phosphatase were from Boehringer Mannheim. HFLC reagents were from J. T. Baker; Chemiaux Co., Phillipsburg, NJ. PKA was prepared as previously described (33). All other chemicals were reagent grade.

Analytical in Situ Phosphorylation—Production and identification of phosphorylated peptides.
of phosphorylated receptors from estrogenized chickens were previously described (6, 19). Briefly, 0.5 g of oviduct slices were placed in 2-mI incubation buffer (25 mM HEPES, 128 mM NaCl, 6.3 mM KCl, 2.8 mM CaCl₂, 1.3 mM MgCl₂, 10 mM glucose, 0.5 mM succinate, 0.5 mM sodium pyruvate, 0.5 mM glutamate, pH 7.4) with 10 μM potassium phosphate, and 0.5 μCi of [³²P]H₂PO₄ in the presence or absence of 100 nM progesterone. After incubation for 30 min at 37°C high followed by 3 min of electrophoresis prepared in homogenization buffer (50 mM potassium phosphate, 10 mM NaMoO₄, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 12 mM 1-thioglycollate, pH 7.0) in an attempt to minimize phosphorylation or dephosphorylation of PRs during isolation.

Immunoprecipitation—Receptors were isolated by immunoprecipitation as previously described (6, 19). Briefly, 0.5 g of oviduct slices were placed in 138 μl of 5-fold concentrated immunoprecipitation buffer (5 × IPB: 2.5 mM NaCl, 0.25 mM Tris, 0.1% NaN₃, 1% Triton X-100, pH 7.5) and 5 μl of PR2 (2 mg/ml) followed by 40 μl of rabbit antiserum IgG and 75 μl of a 50% slurry of protein A-Sepharose in IPB. After washing the immunoprecipitates, proteins were extracted from the beads with SDS-PAGE sample buffer, resolved by 7.5% SDS-PAGE and stained with either silver or Coomassie Blue. For peptide mapping, gels were exposed to XAR-5 film (Kodak) overnight.

In Vitro PR Dephosphorylation—immunoprecipitates, containing 37.5 μl of the protein A-Sepharose beads, were resuspended in 300 μl of alkaline phosphatase buffer (20 mM Tris, 1 mM MgCl₂, 0.1% NaN₃, pH 8.0). Following incubation with alkaline phosphatase for 30 min at 37°C, beads were washed and receptors analyzed by SDS-PAGE.

Analytical Production and Identification of Phosphopeptides—PR bands were cut from gels and digested exhaustively with trypsin as previously described (34). Tryptic peptides were applied to a Vydac C₄ reversed-phase column in 0.1% trifluoroacetic acid in water (eluant A) run at a flow rate of 1 ml/min controlled by a Beckman Instruments model 334 HPLC system. Peptides were eluted with a linear gradient from 0 to 45% acetonitrile in eluant A over 90 min. ³²P-Labeled peptides were identified on-line with a model IC Flo-One beta radioactivity flow detector (Radiomatic Instruments, Inc., Tampa, FL) on a radioactivity detector with a model 120A fluorescence system. Phosphopeptides were identified using the protocol for PKA. The polypeptide-dependent protease (PKA) was a gift from Dr. E. Racker (Cornell University, Ithaca, NY). Calcium/calmodulin kinase type II (42) was a gift from Dr. J. Albracht (University of Texas, Houston).

RESULTS
Regulation of PR Phosphorylation in Situ and In Vitro—In order to analyze PR phosphorylation oviduct slices were incubated with ³²P, in the presence or absence of 100 nM progesterone and receptors isolated by immunoprecipitation followed by SDS-PAGE. Peptide sequencing of PRs isolated from control (Fig. 2, A and B) and PRs (Fig. 2, C and D) from control (Fig. 2, A and C) and progesterone-treated (Fig. 2, B and D) oviduct slices.
It is important to note that these are short, non-steady-state labeling times and therefore do not allow analysis of sites that are fully occupied or turn over slowly. Although PRβ is about 14,000 daltons larger than PRα, the similarity of the maps of the two forms indicates that the predominant sites are within the common amino acid sequences of PRα and not in the unique amino terminus of PRβ. From control slices, two major peaks were seen with mean retention times of 55 (peptide 2) and 76 (peptide 1) min. Progesterone treatment stimulated phosphorylation of these peptides and induced phosphorylation of a third peptide with a retention time of about 81 min (peptide 3).

We next determined the amino acid sequence of the peptides and identified the phosphorylated residues. Tryptic peptides were prepared by direct digestion of immunopurified eluates from progesterone treated oviducts. Following further cleavage with endoproteinase Asp-N (peptides 1 and 2) or endoproteinase Glu-C (peptide 3), peptides were chromatographed as shown in Fig. 3A for progesterone-treated and in Fig. 3B for control oviducts. In each case, a single peak containing most or all of the 32P comigrated with the first peak of a doublet of A_100 nm absorbing material. Doublets such as this are characteristic of phosphopeptides, which comigrate with the 32P, and their cognate dephosphopeptides which elute 1–3 min later in similar HPLC systems (36).

The sequences of each A_100 nm peak were determined by gas-phase sequencing as shown in Table I. Phosphorylated serine residues were identified in the phosphopeptides by (a) the ratio of PTH-Ser (Ser) to PTH-Ser′ (Ser′), the derivative of the dithiothreitol adduct of dehydroserine and (b) release of free 32P. The pmols reported in Table I are derived from sequencing variable aliquots of each peptide and do not reflect total amounts of peptide recovered in each experiment.

![Fig. 1. Regulation of progesterone receptor phosphorylation and dephosphorylation in vivo and in vitro. A, autoradiogram of SDS-PAGE of immunoprecipitated PRs from oviduct slices incubated with ^{[32}P]H3PO4 in the absence (–) or presence (+) of 100 nM progesterone. Arrows show mobility of PRs and PRβ. Panel B, silver stained SDS-PAGE of PRs immunoprecipitated from control (lane 1) or progesterone-injected chickens (lane 2). Immunoprecipitates from injected chickens were incubated with 10, 3.6, and 1.2 units of calf intestinal alkaline phosphatase in lanes 3–5, respectively.](#)

![Fig. 2. Hormonal regulation of PR site-specific phosphorylation. PRs (A and B) and PRa (C and D) were isolated from control (A and C) and progesterone treated (B and D) oviduct slices. Tryptic phosphopeptides were resolved by reversed-phase HPLC and identified by on-line radioactivity detection. The relative elution positions of peptides 1–3 are shown by arrows at the top.](#)

Analysis of peptide 1 from progesterone-treated oviducts is shown in Table I. The dephosphopeptide (retention time (RT) = 46 min) began with Asp-196, ended with Gln-221, and contained Ser-211 at cycle 16 with a high Ser/Ser′ ratio (7.20). The peptide comigrating with the 32P (RT = 44 min) began with Asp-196 and contained Ser-211. For determination of the percentage of free 32P in a separate experiment, the Sequencer was stopped after the indicated cycles to remove portions of the filter. At cycle 16 the release of 32P (86%) and the low ratio of Ser/Ser′ (0.85) indicated that Ser-211 was the phosphorylated residue.

Both forms of peptide 2 (phosphopeptide RT = 32 min, dephosphopeptide RT = 34.5 min) began with Ser-259, ended with Ala-266, and contained Ser-260 (residue 2) and Ser-262 (residue 4) as shown in Table I. Of the three candidate phosphoserines, only cycle 2 showed a low ratio of Ser/Ser′ for the phosphopeptide (1.75) compared to the dephosphopeptide (6.40). While the release of 32P, was moderate at cycle 2 (54%), most of the remaining radioactivity was released by cycle 3 (81%) before the serine in cycle 4. Incomplete release of 32P has been previously reported (43) and may be caused by surrounding residues. Thus, Ser-260 was the predominant site in peptide 2.

As also shown in Table I, both forms of peptide 3 (phosphopeptide RT = 43 min, dephosphopeptide RT = 46 min) began with Leu-527, ended with Glu-534, and contained Ser-530 (residue 4) as shown in Table I. Of the three candidate phosphoserines, only cycle 2 showed a low ratio of Ser/Ser′ for the phosphopeptide (1.99) compared to the dephosphopeptide (7.50) in cycle 4. Furthermore, the majority of the release of 32P occurred at cycle 4 (56%) and not cycle 2 (4%), identifying Ser-530 as the phosphorylated residue. This strategy was also utilized for identification of the doublet peptides and the phosphorylated residues from control oviducts to verify that Ser-211 and Ser-260 were the only detectably phosphorylated residues in peptides 1 and 2 in the absence of progesterone treatment (data not shown). Since so little phosphopeptide 3 was present under these conditions (<2% of the dephosphopeptide 3), the amino terminus was identified by sequencing but the ratios of Ser to Ser′ could not be accurately determined. However, analysis of the release of 32P, indicated that only Ser-530 was phosphorylated.

**Determination of Phosphorylation Stoichiometries**—Stoichiometries were determined by a direct comparison of the doublet absorbance peaks in Fig. 3 since we had demonstrated their relationships by protein sequencing. The values reported below represent the average (greatest range, <5%) of two
acid, this kinase appears to phosphorylate Ser-211. The endogenous oviduct protein kinase phosphorylated the PR predominantly on a tryptic/endoproteinase Asp-N produced peptide that eluted at about 32 min and appeared to be peptide 2. We are not certain of the site phosphorylated by this kinase since peptide 2 contains three serine $Ca^{2+}$/calmodulin-dependent protein kinase type II phosphorylated the PR on one peptide that comigrated with tryptic peptide 2 and on two tryptic peptides different from those seen in situ.

We next tested PKA phosphorylation of the PR as shown in Fig. 4. Of the three hormonally regulated peptides (Fig. 4A), PKA preferentially phosphorylated the highly purified, monomeric receptor on a peptide common to PRa (Fig. 4B) and PRb (Fig. 4C) with a retention time indistinguishable from peptide 3. PKA was the only kinase tested that phosphorylated the receptor on peptide 3. PKA was also the only kinase tested that mimicked the in vivo, hormone-dependent altered mobility seen in Fig. 1B. As shown in Fig. 5, control receptor (lane 1) phosphorylated with PKA (lane 2) exhibited reduced mobility similar to the in vivo hormone-treated receptor (lane 6). Furthermore, the PKA-dependent altered mobility was reversed in vitro with alkaline phosphatase in a dose-dependent manner (lanes 3-5). Finally, the alteration was blocked by preincubation of the PR with an antibody to a synthetic peptide containing the sequences of peptide 3.

We then identified the site phosphorylated by PKA in intact receptor and in the synthetic peptide. Sequence analyses of the phosphorylated and nonphosphorylated forms of the double-digested tryptic/endoproteinase Glu-C synthetic peptide are shown in Table I. The ratio of Ser to Ser was substantially reduced in cycle 2 for the phosphopeptide (1.85) compared to the dephosphopeptide (7.22) but very similar for both forms in cycle 4. Furthermore, $^{32}$P release from in vitro phosphorylation of both the intact receptor (not shown) and the synthetic peptide occurred only at cycle 2. Thus, PKA phosphorylated Ser-528 in vitro.

**DISCUSSION**

In this report we have identified three hormonally regulated phosphorylation sites, common to both PRa and PRb. We determined the regulation of phosphorylation stoichiometry of each site, and evaluated several enzymes for their role in PR phosphorylation/dephosphorylation in vitro.

One site, Ser-530, is in the hinge region between the DNA and hormone binding domains and its phosphorylation is induced about 20-fold by in situ progesterone treatment. Previously we used deletion mutation analysis to identify this region as one of the two transactivation domains of the chicken PR (44), consistent with the role of phosphorylation in functional regulation of transcription factors (45). Furthermore, this site, which is followed by Pro-531, conforms to the Ser-Pro or Thr-Pro (S/T-P) motif postulated to be important for functional regulation of transcription factors (29). The S/T-P motif is present in transactivators of gene expression in much greater abundance than in other general proteins or in DNA binding proteins that are not transcription factors; it is not present in the zinc fingers of DNA binding proteins.

Table III illustrates additional support for the potential importance of hormonal regulation of Ser-530 based on the evolutionary conservation of this S/T-P motif in the hinge region of a large number of proteins belonging to the receptor superfamily including rabbit (46) and human (47) PRs, human glucocorticoid receptor (48) and estrogen receptor (49, 50), rat thyroid receptor isoforms a1 and a2 (51), chicken c-erbA

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### TABLE I

**Identification of PR phosphorylation sites**

Peptides 1–3 from progesterone-treated oviducts were sequenced as described in the text. A synthetic peptide containing peptide 3 was phosphorylated in vitro with PKA and then analyzed.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Amino acid</th>
<th>Position*</th>
<th>D Dephosphopeptide</th>
<th>Phosphopeptide pmol</th>
<th>Phosphopeptide pmol (ser/ser)*</th>
<th>Free &quot;32P&quot; %</th>
</tr>
</thead>
</table>

**Peptide 1**

| 1     | Asp        | 196       | 161                | 210                 | 165              |
| 2     | Ala        | 197       | 152                | 195                 | 195              |
| 3     | Gly        | 198       | 142                | 145                 | 145              |
| 4     | Pro        | 199       | 86                 | 152                 | 152              |
| 5     | Gly        | 200       | 143                | 175                 | 175              |
| 6     | Glu        | 201       | 55                 | 115                 | 115              |
| 7     | Glu        | 202       | 89                 | 71                  | 71               |
| 8     | Gly        | 203       | 118                | 89                  | 89               |
| 9     | Leu        | 204       | 5                  | 165                 | 165              |
| 10    | Ala        | 205       | 79                 | 99                  | 99               |
| 11    | Pro        | 206       | 85                 | 107                 | 107              |
| 12    | Ala        | 207       | 113                | 140                 | 140              |
| 13    | Ala        | 208       | 81                 | 181                 | 181              |
| 14    | Ala        | 209       | 185                | 185                 | 185              |
| 15    | Ala        | 210       | 132                | 198                 | 198              |
| 16    | Ser        | 211       | 36                 | 7.20                | 4.95            |
| 17    | Pro        | 212       | 49                 | 50                  | 90               |
| 18    | Ala        | 213       | 57                 | 76                  | 95               |
| 19    | Ala        | 214       | 81                 | 63                  | 63               |
| 20    | Val        | 215       | 27                 | 35                  | 93               |
| 21    | Glu        | 216       | 25                 |                     |                 |
| 22    | Pro        | 217       | 30                 |                     |                 |
| 23    | Gly        | 218       | 30                 |                     |                 |
| 24    | Ala        | 219       | 33                 |                     |                 |
| 25    | Gly        | 220       | 38                 |                     |                 |
| 26    | Gln        | 221       | 7                  |                     |                 |

**Peptide 2**

| 1     | Ser        | 259       | 44                 | 6.09                | 5.64            |
| 2     | Ser        | 260       | 36                 | 6.40                | 1.75            |
| 3     | Pro        | 261       | 70                 | 218                 | 81              |
| 4     | Ser        | 262       | 23                 | 12.34               | 5.10            |
| 5     | Val        | 263       | 45                 | 96                  |                 |
| 6     | Pro        | 264       | 31                 | 84                  |                 |
| 7     | Ala        | 265       | 24                 | 82                  |                 |
| 8     | Ala        | 266       | 7                  | 51                  |                 |

**Peptide 3**

| 1     | Leu        | 527       | 94                 | 110                 | 0               |
| 2     | Ser        | 528       | 25                 | 7.23                | 5.39            |
| 3     | Phe        | 529       | 74                 | 46                  |                 |
| 4     | Ser        | 530       | 15                 | 7.50                | 1.99            |
| 5     | Pro        | 531       | 48                 | 34                  | 74              |
| 6     | Asn        | 532       | 24                 | 20                  |                 |
| 7     | Gln        | 533       | 20                 | 17                  |                 |
| 8     | Glu        | 534       | 5                  | 13                  |                 |

**Synthetic peptide**

| 1     | Leu        | 527       | 1036               | 464                 | 0               |
| 2     | Ser        | 528       | 270                | 7.22                | 25              |
| 3     | Phe        | 529       | 716                | 224                 | 100             |
| 4     | Ser        | 530       | 221                | 7.20                | 60              |
| 5     | Pro        | 531       | 276                | 147                 |                 |
| 6     | Asn        | 532       | 292                | 120                 |                 |
| 7     | Gln        | 533       | 296                | 70                  |                 |
| 8     | Glu        | 534       | 19                 | 10                  |                 |

* Amino acid position within the PRb.

* Ratio of PTH-serine products from Sequencer.

* Release of "32P, as described under "Materials and Methods".

(52), and the orphan receptors hear1 (53), and humtra 1 and 2 (54). This S/T-P motif is positionally conserved between amino acid residues 50 and 58 carboxyl-terminal to the last cysteine of the second zinc finger. Inspection of these sequences allows the deduction of a putative consensus sequence for phosphorylation consisting of the following residues: non-polar, Ser, or Thr, Pro. This sequence fits the requirements of a recently discovered kinase specific for Ser or Thr followed by Pro (28). Interestingly, this kinase is enriched in proliferating tissues similar to the oviduct of estrogenized chickens used in the present studies. Finally, it is important to note that most other members of the receptor superfamily for

The progesterone receptor is one of the most thoroughly studied steroid hormone receptors. We have also identified two other sites at Ser-211 and Ser-260 in the amino terminus which are modestly regulated by progesterone treatment. Phosphorylation of these sites was about 20% in the absence of hormone and increased 1.5-2-fold by in vivo progesterone treatment. Site 1 was apparently phosphorylated in vitro by the polypeptide-dependent protein kinase (whose specificity is unknown). Site 2, which is part of a PKA consensus sequence, was not phosphorylated in vitro by PKA. The endogenous oviduct protein kinase appeared to phosphorylate peptide 2, on or near site 2, while calmodulin kinase II potentially phosphorylated peptide 2. Although these kinases phosphorylate the PRs in vitro, we do not know whether they play a role in receptor phosphorylation in vivo. It is notable that sites 1 and 2, analogous to site 3, are dephosphorylated in response to hormone, or are constitutively filled or turn over slowly would not be labeled sufficiently for analysis. This is in contrast to studies in cell lines that have analyzed receptors labeled under steady-state conditions. In combination with the previous indirect methods used for site localization, these experimental differences may account for the discrepancies in estimates in the total stoichiometry, number, and location of phosphorylation sites in steroid hormone receptors.

The progesterone receptor is one of the most thoroughly studied receptors regarding the number of phosphorylation sites. Initial reports that provided the framework for the previous indirect methods. In combination with the previous indirect methods used for site localization, these experimental differences may account for the discrepancies in estimates in the total stoichiometry, number, and location of phosphorylation sites in steroid hormone receptors.
Progesterone Receptor Phosphorylation Sites

Amino and/or carboxyl termini and would result in multiple peptides in two-dimensional thin-layer electrophoresis/chromatography. However, these complex HPLC peaks certainly represent single sites since the second proteolytic digestion converts all of the \(^{32}P\) associated with each peak into a single, sharp peak of one peptide with single amino and carboxyl termini. HPLC mapping of PRs lead to the suggestion that three to five peptides were phosphorylated in the absence of hormone treatment. Hormone stimulated phosphorylation of several peptides in the chicken PR (24) and induced phosphorylation of two peptides in the human PR (25). While \(^{32}P\) peaks obviously represent different peptides, they do not necessarily represent unique sites.

We were additionally interested to identify kinases that may phosphorylate the PR. In work to be reported elsewhere we showed in transient transfections that stimulation of PKA mimicked progesterone-dependent induction of target gene expression while inhibition of PKA blocked progesterone action. In the present in vitro studies we showed that PKA mimicked the in vivo, hormone-dependent altered mobility seen by SDS-PAGE. This mobility shift could be reversed with alkaline phosphatase similar to that observed for the in vivo phosphorylated receptor. Also, the shift could be blocked by preincubation of the receptor with an antibody to a synthetic peptide containing the sequences of peptide 3. PKA preferentially phosphorylated peptide 3 containing the hormone-induced Ser-530. However, peptide sequencing indicated that PKA did not phosphorylate the PR on Ser-530 but instead on nearby Ser-528 which is part of a PKA consensus.

In view of these results it seems likely that under physiological conditions PKA is not a direct mediator of progesterone action in vivo. Our observations on PKA regulation of PR-mediated transcription may represent the involvement of PKA in a cascade system regulating other kinases and phosphatases (see Refs. 57 and 58) which then regulate phosphorylation of Ser-530. Such an indirect role is consistent with the observation that all three hormone-regulated PR sites contain the S-P motif, a preferred substrate of the proline-directed protein kinase. Alternatively, since the highly purified, monomeric receptors used in these experiments may not be in the appropriate conformation and are not associated with the appropriate proteins (such as HSP90), our in vitro results may not reflect events that occur in vivo. Finally, PKA regulation of PR-mediated transcription in vivo may be an example of the role of negative charge in producing a protein surface, conformation, or charge cluster required for transactivation of target genes (45). Thus, elevation of PKA in vivo may result in direct phosphorylation of Ser-528 which could substitute for the hormone-induced physiological phosphorylation of Ser-530 by providing the requisite charge within a specific activation domain.

The role of phosphorylation in the regulation of the activity of transcription factors has been addressed only recently by direct experiments. Some of the clearest studies performed to date have been with SV40 T antigen (59-63), the CAMP response element binding protein (64), serum response factor (65), yeast heat shock factor (66, 67), DNA topoisomerase II (68, 69), and E4F (18). The most definitive work on members of the receptor superfamily of transcription factors showed, by site-directed mutagenesis, that phosphorylation of identified Ser residues in the amino-terminal region of chicken erbA regulates biological activity. Thus, the precedent clearly exists for regulation of receptor-mediated transcription by phosphorylation. Identification of PR phosphorylation sites should now allow definitive determination of the role of site-specific phosphorylation in functional regulation of the chicken PR.

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