Serine 157, a Retinoic Acid Receptor α Residue Phosphorylated by Protein Kinase C in Vitro, Is Involved in RXR-RARα Heterodimerization and Transcriptional Activity*

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Retinoic acid (RA) regulation of cellular proliferation and differentiation is mediated, at least in part, through two related nuclear receptors, RAR and RXR. RA-induced modulation of gene expression leads generally to cellular differentiation, whereas stimulation of the protein kinase C (PKC) signaling pathway is associated with cellular proliferation. Pursuant to our discovery that prolonged activation of PKCs induced a strong decrease in RA responsiveness of a retinoid-inducible reporter gene, we have further investigated the connections between these two signaling pathways. We demonstrate that PKC isoforms α and γ are able to phosphorylate human RARα (hRARα) in vitro on a single serine residue located in the extended DNA binding domain (T box). The introduction of a negative charge at this position (serine 157) strongly decreased hRARα transcriptional activity, whereas a similar mutation at other PKC consensus phosphorylation sites had no effect. The effect on transcriptional activation was correlated with a decrease in the capacity of hRARα to heterodimerize with hRXRα. Thus hRARα is a direct target for PKCα and γ, which may control retinoid receptor transcriptional activities during cellular proliferation and differentiation.

External stimuli (neurotransmitters, hormones, mitogens) activating G-protein-coupled receptors and growth factor receptors induce the activity of phospholipases, which in turn generate membrane lipid metabolites. Among them, diacylglycerol (DAG) and others are able to modulate cellular protein kinase C (PKC) activities needed for sustained cellular responses (1, 2). The PKC superfamily contains to date 11 isoforms encoded by 10 genes. The isoforms can be classified into three groups based on sequence homologies and biochemical properties: conventional PKCs (isoforms α, β1, β2, and γ) are DAG- and calcium-dependent kinases; novel PKCs (isoforms δ, ε, η, θ) are dependent only on DAG; and a third group of atypical PKCs (isoforms ξ, ι, λ) is unresponsive to DAG and calcium. The μ isoform (mouse protein kinase D) is a high molecular weight enzyme with specific properties such as a transmembrane domain, but it can be considered an atypical PKC in view of its very low affinity for DAG (for review, see Refs. 3 and 4). All of these kinases need negatively charged phospholipids (phosphatidylserine) to exert their phosphorylating activities, triggering major cellular events such as differentiation and proliferation. Although a clear physiological role for each isoform has not been established definitively, the existence of these isotypes with unique subcellular and tissue distribution suggests a specialized role for each isoform (for review, see Ref. 5). Most notably, gene transfer experiments have underlined a role of these protein kinases in cellular proliferation and tumour growth progression.

Nuclear receptors are ligand-dependent transcription factors that control a wide range of cellular events, including cellular proliferation and differentiation. Several lines of evidence point to a direct role of the PKC signaling pathway in modulating the transcriptional activity of some members of this superfamily. The thyroid hormone receptor appears to be a target for PKC (6) as well as the human vitamin D receptor (hVDR). hVDR is phosphorylated by PKCβ in the DNA binding domain (DBD), and phosphorylated serine (Ser-51) is important for hVDR transactivation properties (7, 8). Regulation of other nuclear receptors (glucocorticoid receptor, peroxisome proliferator-activated receptor, estrogen receptor) through activation of the MAP kinase cascade has also been documented and suggests that PKC, as an activator of the MAP kinase pathway, might participate indirectly in the control of the transcriptional activity of these nuclear receptors (9–13).

Our previous experiments pointed to a role of PKC in the regulation of hRARα transcriptional activity. They indicated that chronic treatment of COS-7 cells with TPA led to the specific inhibition of the activity of a retinoid-inducible reporter gene. We determined that hRARα is a substrate for PKC in vitro, and preliminary results suggested that two to four phosphorylation sites occurred out of the ligand binding domain (LBD) of this nuclear receptor (14). In light of these results, we addressed in this study several questions to elucidate further the role of PKC isoforms in the regulation of the transcriptional activity of hRARα. We characterized PKC isoforms for their capacity to phosphorylate hRARα in vitro and identified the target amino acid. Mutation of the phosphorylated serine affected hRARα transcriptional activity, and the molecular basis for the observed inhibition was investigated. Our results support the hypothesis that PKCα and PKCγ are direct regu-
lators of hRARα transcriptional activity by altering its ability to dimerize with RXRs.

**EXPERIMENTAL PROCEDURES**

**Materials**—TPA and atRA were obtained from Sigma (St. Quentin Fallavier, France). Purified rat brain PKC was purchased from Calbiochem-Novabiochem Corp. (Meudon, France). [γ-32P]ATP (3,000 Ci/mmol), the ECLPLUS detection kit, and polyvinylidene difluoride transfer membranes were purchased from Amersham Pharmacia Biotech (Leu Ulis, France). DNA restriction and modifications enzymes were from Pro- mega (Charbonnieres, France). Site-directed mutagenesis oligonucleotide primers were carried out using the QuickChange system from Stratagene (San Diego, CA). Polyethyleneimine (ExtGen 500) was from EuroMedex (Souffleweyerhein, France). Oligonucleotides were purchased from Eurogentec (Le Sart-Tilman, Belgium).

**Plasmids**—Constructs containing either the wild-type (wt) hRARα and wt hRXRα cDNAs subcloned into pSG5 (Stratagene, La Jolla, CA) or PEQ9 (BmiGene GmbH, Dusseldorf, Germany) were used. Site-directed mutagenesis oligonucleotide primers were used in this study (parentheses indicate the new restriction site, mutations are indicated in bold characters).

S150G: 5′-cgccgccgctacgaagagacagc3′ (SacI);
S115D: 5′-cgccgccgctacgaagagacagc3′ (SacI);
S154A: 5′-gaagcttgcagctgaggaagagc3′ (PvuI);
S154A, 157A: 5′-gaagcttgcagctgaggaagagc3′ (PvuI);
S154A, 157D: 5′-gaagcttgcagctgaggaagagcgctgtgagaaacggacgaaacaagaagc3′ (PvuI);
S154D: 5′-actggaacagtcggctcgaagagc3′ (BstI);
S157D: 5′-gtcgagctgctgaggaagagc3′ (PvuI);
S157D, 154D: 5′-gtcgagctgctgaggaagagc3′ (PvuI); and
S157D: 5′-gtcgagctgctgaggaagagc3′ (PvuI).

**Transfections**—HeLa cells were cultured as monolayers in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum. Cells were treated with indicated TPA and/or retinoic acid to a final concentration of 10−5 M and 10−8 M, respectively. Transfections were carried out using the polyethyleneimine coprecipitation method as described (17). The luciferase assay was performed as described (15).

**Cell Culture and Transfections**—HeLa cells were cultured as monolayers in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum. Cells were treated when indicated with TPA and/or retinoic acid to a final concentration of 10−5 M and 10−8 M, respectively. Transfections were carried out using the polyethyleneimine coprecipitation method as described (17). The luciferase assay was performed as described (15).

**Receptor Purification and Phosphorylation Analysis**—A detailed procedure has been published elsewhere (18). Briefly, His6-hRARα was overexpressed in *Escherichia coli* and purified to homogeneity by nickel-imobilized affinity chromatography. The purified polypeptide was phosphorylated and purified further by 8% SDS-PAGE. Gel slices containing the phosphorylated receptor were desiccated and rehydrated with trypsin digestion buffer, and trypsin was added to a 1:50 (w:w) ratio. Peptides were extracted and purified on a C18 column and subjected to Edman degradation reaction for sequencing. Phosphoamino acid standards have been described in Ref. 14.

**RESULTS**

**hRARα Is Phosphorylated by PKCα and PKCy on Serine Residues in Vitro**—We reported previously that one or two amino acids in the hRARα sequence located outside of the LBD were phosphorylated by PKCs purified from rat brain (14). We thus determined which isoforms of PKC were present in these commercial preparations by Western blotting analysis using a panel of isozyme-selective monoclonal antibodies. We found that rat brain extracts contained exclusively the PKCα and γ isoforms, with trace amounts of the μ isofrom (Fig. 1A). Using this protein kinase mixture in an *in vitro* phosphorylation reaction with purified His6-tagged hRARα as a substrate, we found that this receptor was a substrate for PKCα, as reported previously (Fig. 1B). PKCs were able, in these conditions, to display autophosphorylating activity as documented previously (for review, see Ref. 21), but no kinase activity was detected in purified hRARα preparations. The PKCα-catalyzed hRARα phosphorylation was inhibited by GF109203X, a specific but general inhibitor of PKCs, and by an inhibitor specific for α, β, and γ isoforms (G66976; Ref. 22). G66976 strongly inhibited PKC-directed phosphorylation of hRARα, but phosphate incorporation was still detectable, suggesting that PKCy may display some weak phosphorylating activity in these conditions. From these experiments, we conclude that PKCy is the most active isozyme phosphorylating hRARα in *vitro*.

Phosphorylated hRARα was gel purified and submitted to acid hydrolysis to determine which type of amino acid was phosphorylated in these conditions. Products were analyzed by thin layer chromatography and identified by comigration with phosphoamino acid standards (Fig. 1C). Only phosphoserine was detected in these conditions, suggesting that hRARα is phosphorylated on serine residues by PKCy.
phosphorylated by PKC only on serine(s) located outside of the LBD.

Identification of Serine Residues Phosphorylated in Vitro by PKCα and PKCγ—To identify hRARα amino acids phosphorylated by PKCα and PKCγ in vitro. His6-tagged hRARα was purified to near homogeneity from E. coli extracts using NiTA affinity chromatography and incubated with purified rat brain PKCs and [γ-32P]ATP, in the presence of a PKC inhibitor (100 nM Go6976). Phosphorylated proteins were analyzed by 8% SDS-PAGE, and gels were either silver stained (left panel) or autoradiographed (right panel). Panel C. PKCα and PKCγ phosphorylate hRARα on serine residues. hRARα was phosphorylated by rat brain PKCs and gel purified as in panel B. The polypeptide was submitted to total acid hydrolysis, and reaction products were separated by thin layer chromatography and identified by comigration with phosphoamino acid standards.

and Ser–115, Ser–154, Ser–232, and Ser–388 into an alanine (A) to determine whether these serine residues could be minor phosphorylation sites undetected in our phosphopeptide mapping experiments. His-tagged wt and mutated hRARα were expressed in bacteria, purified by immobilized metal affinity chromatography, and tested for their ability to serve as a substrate for PKC. Phosphorylation reactions were carried out as described above, using an identical amount of each receptor derivative, and products were subjected to SDS-PAGE. Receiver concentrations were controlled by silver staining (Fig. 3, left panels) of gels, which were then dried and autoradiographed (Fig. 3, right panels). We found that only mutation of Ser–157 severely compromised PKC-catalyzed phosphorylation, whereas all other substitutions designed to inactivate other consensus sequences had no significant effect on the phosphate content of hRARα. Residual phosphate incorporation was, however, observed with the S157A mutant, suggesting that other sites may represent poor substrates for PKC. Alternatively, this residual activity might result from contaminating, unidentified protein kinases in the rat brain extract. Thus Ser–157 appeared to be the major target for PKC in vitro, and these data support both peptide mapping results and our previous conclusion that putative phosphorylation site(s) lie outside of the LBD (14). Ser–157 is located in the DBD of the receptor, in a region lying COOH-terminally of the second zinc finger motif. Sequence alignment between RAR and RXR isoforms showed that Ser–157 is found only in RARα and RARβ (Fig. 4) and is located in the so-called T box region, which has been reported.
to be important for dimerization and DNA binding activities of several nuclear receptors (23, 24).

Converting Serine 157 into Aspartic Acid Decreases Transcriptional Activity of hRARα—To examine the role of this phosphorylatable amino acid in regulating the transcriptional activity of hRARα, we constructed eukaryotic expression vectors encoding Ser to Ala or Gly, and Ser to Asp hRARα mutants, either to prevent constitutive phosphorylation by PKC (Ser to Ala/Gly mutants) or to mimic the net charge increase brought by phosphorylation (Ser to Asp mutants). As for in vitro experiments, we deliberately mutated all potential phosphorylation sites for PKC (Ser-115, Ser-157, Ser-232, Ser-388, and Ser-452) and compared the activity of Ser to Ala and of Ser to Asp mutants with that of wt hRARα. HeLa cells express low levels of RARs and RXRs which are able to activate prototypic retinoid response elements such as the direct repeat found in the RARβ2 gene promoter (DR5). To avoid any interference with endogenous receptors, we used the luciferase reporter gene driven by the synthetic, palindromic thyroid response element TREpal, which is also activated by retinoids but necessitates high levels of receptors which are only achieved upon transfection of RAR and/or RXR expression vectors (17, 25). We thus monitored the transcriptional activity of Ser to Ala and of Ser to Asp mutants in the presence of atRA as an inducer at a 10^{-6} M final concentration (Fig. 5). The basal level of luciferase activity measured in the presence of wt hRARα or mutant receptors did not fluctuate significantly, suggesting that these receptors have a similar affinity for nuclear corepressors.

In vitro protein-protein interaction assays revealed that all of these receptors bound to the nuclear corepressor SMRT with a wt affinity. The addition of atRA to the culture medium leads typically to a 5–10-fold increase in luciferase activity: only two mutations affected the ability of hRARα to respond to atRA, S115G and S157D, although both receptors were expressed at levels comparable to that of wt hRARα. Because Ser-115 is not phosphorylated by PKCs (Fig. 3), and S115D displayed a wt activity, it is likely that the observed decreased activity results from structural alterations of the DBD and not from direct phosphorylation. Ser-115 is indeed located between the two zinc fingers of hRARα, and mutations at this position in the hVDR sequence

\[3 \text{ M.-H. Delmotte and P. Lefebvre, unpublished data.} \]
(S51) were proposed to alter the α-helical structure of this region (8). A decrease of at least 50% was observed in atRA-induced luciferase activity in cells overexpressing S157D, whereas S157A retained wt activity, consistent with our working hypothesis of a PKC-mediated inactivation of hRARα. The latter observation also suggests that no phosphorylation of Ser-157 occurs in nonstimulated cells, in agreement with a previous report (27). We also note that this decrease in activity is similar to that observed upon chronic treatment of cells with TPA (14), and therefore identify Ser-157 as a functional substrate for PKCs.

Phosphorylation of Serine 157 Decreases the Ability of hRARα to Heterodimerize with RXR—The effects of the Ser-157 mutation on RAR properties were investigated: the ability of the mutated receptor to bind natural and synthetic ligands, its subcellular localization in response to ligand as well as its half-life, its interaction with nuclear corepressor (SMRT) and coactivators (SRC-1, RIP140) were tested. None of these assays revealed functional alterations of the receptor.3 The ability of the receptor to interact with RXR in a ligand-dependent manner in the absence of DNA was quantified in glutathione S-transferase pull-down experiments (Fig. 6). A Sepharose GST-RXR affinity matrix was used as a bait for35S-labeled wt hRARα and receptor mutants S157A and S157D, in the presence or not of 1 μM atRA. Similar amounts of receptors were used in each condition and high salt washes allowed to monitor the ligand-induced stabilization of RAR-RXR interaction. Heterodimer formation was stimulated 3-fold for wt hRARα and the S157A mutant (Fig. 6A). S157D displayed a slightly but significantly decreased constitutive dimerization (in the absence of atRA) and a 2-fold reduction in its ability to dimerize with RXR in the presence of atRA. This result was confirmed using an independent assay in which phosphorylated or native His-tagged RAR was immobilized on a hydrophobic matrix (8). 35S-Labeled RXR was then incubated, in the presence of 1 μM atRA, with this matrix, and specifically bound receptors were quantified (Fig. 6B). RAR phosphorylation by PKC, in conditions where more than 80% of the receptor was phosphorylated by PKCs, led to a 50% decrease in RXR retention on this matrix, in agreement with results generated by the glutathione S-transferase pull-down assay. Thus to a 50% decreased transcriptional activity is associated with a decreased ability to form a dimer with hRXRα, strongly suggesting that PKC-catalyzed post-translational modification of hRARα is controlling the ability of RAR to heterodimerize with RXR.

**DISCUSSION**

Modulation of the phosphorylation state plays an important role in the reversible control of the activity of many proteins, including nuclear receptors. In the case of hRARα, heterodimerization properties have been shown to be altered by protein phosphatases 1 and 2A (15) and the NH2-terminal function AF-1 to be activated through the proline-directed Cdk7 (27). We now show that dimerization of hRARα with hRXRα can be modulated by PKC-dependent phosphorylation. Several potential phosphorylation sites by PKCs are located along the hRARα sequence. Phosphopeptide mapping studies as well as mutagenesis of hRARα identified Ser-157 as the major target for PKCα and PKCγ isoforms (Figs. 2 and 3). This amino acid is located in the T box of hRARα, which, together with the A box, are part of a larger region (hinge domain) that is organized into an α-helical structure in the RXR-T-R DBD dimer (23). Molecular modeling of RXR-RAR dimers suggested that the T box, forming a loop interrupted by an α-helical turn perpendicular to the DNA axis, engages dimerization contacts with the RXR DBD. Interestingly, Ser-157 neighbors amino acids that, in RXR-T-R and RXR-VDR dimer structures, establish salt bridges with the RXR DBD (Asp-69 and Glu-69, respectively). Thus phosphorylation of Ser-157 may introduce strong conformational constraints on this dimerization region and, as observed, decrease the relative affinity of RAR DBD for RXR DBD and inhibit dimerization. Alternatively, phosphorylation of Ser-157 may perturb the relative orientation of RAR DBD and LBD as suggested for VDR (24) and in turn modify the orientation of the strong dimerization interface located in the hRARα LBD (8). Protein kinases C are a family of Ser/Thr kinases whose activities are involved in the regulation of complex biological responses such as differentiation, proliferation, and apoptosis. They are organized in two domains, a COOH-terminal catalytic domain and an NH2-terminal regulatory domain. Their tissue distribution is in most cases ubiquitous, with the exception of PKCγ, which is expressed specifically in the brain and spinal cord. Response to agonists (hormones, neurotransmitters, growth factors) is mediated through a transient short term production of DAG, essentially through phosphatidylinositol 4,5-bisphosphate hydrolysis. A more sustained response may be observed with phosphatidylethanolamine hydrolysis or phorbol ester treatment. The basic model of PKC activation suggests that PKC translocation to the plasma membrane is the main event regulating this signaling cascade. However, PKC is often found, irrespective of its activation state, in the particulate fraction (membranes, organelles, and nuclei) of cul-
After extensive washing to remove unbound ATP, 10^6 cpm of 35S-labeled substrates for PKCs: LIM-containing proteins (29), p53 (30), established. However, in these types of experiments, S157A purified wt hRAR protein input lane as a reference (which represents 20% of total input) and analysis. Results are shown as the average of three independent experiments. SNAP-25. These data support the view that these two proteins kinases, and c-Jun NH2-terminal kinase (JNK). PKC down-regulation has been reported to block completely JNK activation by phorbol esters in HeLa cells (41), and MAP kinase overexpression in COS cells did not modify the phosphorylation pattern of hRAR α (27). No increase in MAP kinase activity was detected in TPA-treated HeLa cells, thus ruling out a potential involvement of these two protein kinases in the observed inactivation of hRAR α.

Furthermore, additional cross-talk processes can be also considered based on the nongenomic effects of steroid and of others ligands for nuclear receptors. For example, vitamin D3 has been shown to activate the MAP kinase signaling cascade through activation of PKC (42) and is able to induce subcellular redistribution of calcium-dependent PKCs. Tamoxifen, a therapeutic/chemopreventive nonsteroidal antagonist of estrogen receptor, induces PKC translocation to the membrane and down-regulation of this enzyme through oxidative stress (43). Finally, retinoids, estrogens, and vitamin D3 treatment of cells modulate the rate of expression of PKC isoforms (26, 44–46), underlying a complex network of intertwined, and often antagonistic, signaling pathways eventually controlling cell differentiation, growth, and death.

In conclusion, our findings establish that PKC isoforms are able to phosphorylate hRAR α and control its dimerization properties, very likely through T box conformational alteration. The observed inhibition may be of importance in differentiation and proliferation processes during which sustained activation of PKCs is necessary to induce a full biological response to external stimuli.

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