TFIIH Interacts with the Retinoic Acid Receptor γ and Phosphorylates Its AF-1-activating Domain through cdk7*

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Retinoic acid receptor γ (RARγ) is phosphorylated in COS-1 cells at two conserved serine residues located in the N-terminal region (serines 77 and 79 in RARγ1 and serines 66 and 68 in RARγ2) that contains the activation function AF-1. These serines are phosphorylated in vitro by cdk7, a cyclin-dependent kinase associated to cyclin H and MAT1 in the CAK complex (cdk7-cyclin H-MAT1), that is found either free or as a component of the transcription/DNA repair factor TFIIH. RARγ is more efficiently phosphorylated by TFIIH than by cdk7 and interacts not only with cdk7 but also with several additional subunits of TFIIH. RARγ phosphorylation and interaction with TFIIH occur in a ligand-independent manner. Our data demonstrate also that phosphorylation of the AF-1 function modulates RARγ transcriptional activity in a response gene-dependent manner.

The pleiotropic effects of retinoids are transduced by two nuclear receptor families, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs), that are ligand-dependent transregulators belonging to the nuclear receptor superfamily (1–4). RARs are activated by all-trans and 9-cis retinoic acid, whereas RXRs are activated by 9-cis retinoic acid only. There are three RAR (α, β, and γ) and three RXR (α, β, and γ) isotypes, and for each isotype there are at least two main isoforms that differ in their N-terminal region (1, 5, 6).

As do other members of the nuclear receptor superfamily, RARs and RXRs exhibit a conserved modular structure with six variably conserved regions (A to F) (Fig. 1) (1, 5). The N-terminal A/B region of RARs contains a ligand-independent transcriptional activation function, AF-1 (7, 8). Although the B regions of the three RAR isotypes are moderately conserved, their A regions are unrelated and differ for each isoform of a given RAR isotype (5). The highly conserved C region encompasses the central DNA binding domain. The function of region F, if any, is unknown. Region E is more complex, as it contains the ligand binding domain, a dimerization interface, and the ligand-dependent transcriptional activation repression domain AF-2 (1, 9). The activity of AF-2 is entirely dependent on the integrity of a conserved sequence referred to as the AF-2 AD core, located in α-helix 12 at the C-terminal end of the ligand binding domain. Ligand binding induces a major conformational change that includes helix 12 and creates a new surface for coactivator binding while corepressors are released, thus resulting in a transcriptional-competent nuclear receptor relayed to the transcriptional machinery and the chromatin template (1, 10–12). The AF-2 and AF-1 activities synergize with each other in a response element- and promoter context-dependent manner (1, 8, 13).

RARs and RXRs are phosphoproteins (14–16), and their phosphorylation involves several kinases. RARα can be phosphorylated in its AF-1-containing B region by the cyclin-dependent kinase cdk7 (14), which together with MAT1 and cyclin H forms the CAK complex that is found either free or as a component of the general transcription/DNA repair factor TFIIH (17–20). This phosphorylation, which results from an interaction with cdk7, is crucial for RARs transcriptional activity and modulates its ligand-induced degradation by the ubiquitin-proteasome pathway.2 RARα can also be phosphorylated by protein kinase A at a residue located in the ligand binding domain (15), and this phosphorylation is required for differentiation of mouse embryonal carcinoma F9 cells into parietal endoderm-like cells upon RA and cAMP treatment (13). Similarly, RXRα was found to be phosphorylated in its N-terminal A/B region and shown to be hyperphosphorylated in the same region by c-Jun N-terminal kinases upon UV activation (16).

Mutations of putative phosphorylation sites located in the AF-1 domain of mRARγ2 were found to prevent the RA-induced differentiation of F9 cells (13), thus indicating that RARγ2 phosphorylation in this domain could be required for this differentiation. Moreover, phosphorylation in the same domain was recently shown to be crucial for the ligand-induced degradation of RARγ by the ubiquitin-proteasome pathway.2 Because of our previous demonstration that RARα can be phosphorylated by cdk7 present within TFIIH, we assumed that RARγ could also be phosphorylated by TFIIH. In the present study, we demonstrate that the B region of the two major human or mouse RARγ isoforms, RARγ1 and RARγ2, are phosphorylated in a ligand-independent manner, by the cyclin H- and MAT1-dependent protein kinase cdk7. We also show that phosphorylation of RARγ is more efficient when cdk7 is present...


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within TFIIH. In addition, we reveal the existence of multiple RAR-γ-TFIIH interactions that involve not only cd7k but also additional subunits of TFIIH. Finally, phosphorylation of the A/B region was found to modulate RAR-γ-induced transcription in a response gene-dependent manner.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Construction of Receptor Mutants—**The pSg5-based expression vectors for human (h) RAR-γ, mouse (m) RAR-γ, mRAR-γ, and mRAR-γ AAb were previously described (7, 21–23). hRAR-γ/ST76A, ST77A, and ST77/A/ST79A in pSg5 were constructed by double PCR amplification according to Ho et al. (30) to generate an MscI/SacI fragment containing the appropriate mutation. The external oligonucleotides were 5′-CAGAATTCTAGTCTGTGAAGTCAATGACA-3′ and 5′-CGGGATCCTCAGGCTGGGGACTTGACG-3′ and subcloning the amplified product into EcoRI-BamHI-digested hRAR-γ/pET3d. mRAR-γ/ST76A/S78A in pSg5 was constructed by subcloning the KpnI/MscI fragment from RAR-γ/S76A/S78A in pD002A (13) into the same sites of pSg5. mRAR-γ/ST76A in pSg5 was constructed by exchanging the MscI/SacI fragment of the mRAR-γ expression vector with a MscI/SacI-digested PCR-amplified fragment encoding amino acids 223–410. mRAR-γ/ST76A/S78A/S79A/AAB was constructed by double PCR amplification reaction to generate a MscI/SacI fragment containing the appropriate mutation. The external nucleotides were 5′-CAGGCAAGCTTCCGGACCAATTGCACT-3′ and 5′-CTTGGGGAAATACGAGGCTC-3′.

Vectors encoding the chimeric proteins hRAR-γ(A/B)-ER(C) and Gal4-mRAR-γ(DEF) in pSg5 were as described (8). hRAR-γ/1(WT) and aAB were mutagenized at serine 77 and/or 79 were created by cloning the MscI/SacI fragment from pSGS hRAR-γ into the same sites of the chimeric construct.

The procaryotic vectors pET3d hRAR-γ/1WT, ST77A, ST79A, and ST77A/ST79A were constructed by subcloning the Nco1-BamHI fragments from the corresponding pSg5 vectors into the same sites of pET7d. Escherichia coli selected, over expressed, and purified proteins were mutated as described (28). Purified hRAR-γ/1WT and hRAR-γ/1AAB were gifts from H. Gronemeyer (IGBMC, Illkirch, France).

The expression vectors for cd7k and cd7k7m were as described (14). Recombinant cd7k and CD77 were produced and purified from baculovirus-infected Sf9 cells as described (19, 26). Highly purified TFIHH hydroxyapatite fractions were prepared from HeLa cells (27). Baculoviruses allowing the expression of single subunits of the TFIIH core (XPB, XPD, MAT1) were as described (17). Baculovirus encoding for hRAR-γ was expressed as His-tagged fusion protein was constructed in the pVL1392 vector (20 ng, Upstate Biotechnology Inc., Lake Placid, NY). Phosphoamino acids were resolved by SDS-PAGE, electrotransferred to nitrocellulose filters, and visualized by autoradiography or by chemiluminescence after reaction with specific antibodies (14, 15).

For phosphorylation in transfected cells, COS-1 cells were transfected with wild type or mutated RAR-γ expression vectors using the standard calcium phosphate procedure and labeled with [32P]orthophosphate as described (15). Whole cell extracts were immunoprecipitated with hRAR-γ AAb and resolved by SDS-PAGE, and after electrophoretic transfer, the proteins were revealed by autoradiography and immunoblotting. Two-dimensional phosphoamino and tryptic phosphopeptide separations were carried out on thin layer cellulose plates using the Hunter thin-layer electrophoresis (HTLE) system as described (15).

**RESULTS**

**Both Human and Mouse RAR-γ Overexpressed in COS-1 Cells Are Phosphorylated in Their N-terminal A/B Region, whereas Mouse RAR-γ Is Additionally Phosphorylated in Its F Region—to determine whether wild type human RAR-γ (hRAR-γ/1WT) is a phosphoprotein, COS-1 cells were transfected with the corresponding expression vector and labeled with [32P]orthophosphate in the absence or presence of RA (10−7 M). Whole cell extracts were immunoprecipitated with a RAR-γ-specific monoclonal antibody and resolved by SDS-PAGE, and the phosphorylated proteins were analyzed either by autoradiography or by immunoblotting. hRAR-γ was phosphorylated irrespective of the addition of RA to the culture medium (Fig. 2A, lanes 1 and 2). Phosphoamino acid analysis indicated that phosphorylation was restricted to serine residues (Fig. 2B).

Tryptic phosphopeptide mapping yielded 3 phosphopeptides a, b, and c (Fig. 2C, panel 1). To characterize these phosphopeptides, COS-1 cells were transfected with hRAR-γ/1, deleted for the A/B region (hRAR-γΔA/B) and labeled with [32P]orthophosphate. hRAR-γAAB was not phosphorylated (Fig. 2A, lane 3), suggesting that phosphopeptides a, b, and c are located in the A/B region. Similarly, transfection in COS-1 cells of a chimeric construct expressing the A/B region of hRAR-γ fused to the DNA binding domain (C) of the human estrogen receptor (ER) showed that the corresponding chimeric protein, hRAR-γ(A/B)-ER(C), was phosphorylated (Fig. 2A, lane 8) and yielded the three phosphopeptides a, b, and c (Fig. 2C, panel 2).

Because the above data suggested that the A/B region of hRAR-γ/1 is a target for phosphorylation, the three series belonging to potential phosphorylation sites for proline-directed kinases were individually mutated to alanine. The corresponding mutants, hRAR-γ/1WT, S76A, S77A, and S77A/ST79A (Fig. 1C) were transfected in COS-1 cells and analyzed by phoshoamino acid analysis.
phosphopeptide mapping. hRARy1S77A, S79A, and S77A/S79A exhibited differences in their phosphorylation level (Fig. 2A, compare lanes 4–7), and phosphopeptide mapping analysis revealed that hRARy1S77A lacked phosphopeptide a (Fig. 2C, panel 3), whereas hRARyS79A and hRARyS77A/S79A lacked all three phosphopeptides (Fig. 2C, panel 4, and data not shown). Similar results were obtained with the chimeric construct hRARy1(A/B)-ER(C) in which serines 77 and 79 were mutated (data not shown). However, phosphorylation of the hRARy1S76A mutant was similar to that of hRARy1WT (data not shown), indicating that serine 76 is not phosphorylated.

Altogether, our results indicate that hRARy1WT is phosphorylated at serines 77 and 79. They also indicate that phosphopeptide a contains serine 77, whereas phosphopeptides b and c contain serine 79. These two b and c peptides may be partial digestion products due to the presence of low efficiency sites for trypsin cleavage in human RARy1 (37). In addition, the fact that mutation of serine 79 eliminates all phosphopeptides suggests that phosphorylation of serine 77 depends on that of serine 79 (37). Finally, as both serines 77 and 79 are located in the same tryptic peptide, it is not excluded that peptides a, b, and c are phosphoisomers, spot “a” representing the phosphopeptide a contains serine 77, whereas phosphopeptides b and c contain serine 79. These two b and c peptides may be partial digestion products due to the presence of low efficiency sites for trypsin cleavage in human RARy1 (37).

The phosphorylation sites that are located in the B region of RARy are conserved between human and mouse and identical in the RARy1 and RARy2 isoforms (21, 23) (Fig. 1). In contrast, human and mouse regions F are different (Fig. 1) (21). Therefore, the two mouse RARy isoforms (mRARy1 and mRARy2) were overexpressed in COS-1 cells and labeled with [32P]orthophosphate. Both receptors were phosphorylated in a RA-independent manner (Fig. 3A, lanes 1–4). Tryptic phosphopeptide mapping of mRARy1 yielded, in addition to phosphopeptides a, b, and c described above for hRARy1, two other phosphopeptides, d and e (compare Fig. 3B, panel 1 to Fig. 2C, panel 1). The same d and e peptides were present in the tryptic digest of mRARy2, whereas phosphopeptides a’ and b’ differed from phosphopeptides a and b of RARy1, and phosphopeptide c’ was not seen (Fig. 3B, compare panels 1 and 2; see also panel 6), most probably because regions A of these two RARy isoforms are unrelated in their sequence (23). As expected, mRARyAB was less phosphorylated than mRARy2WT (Fig. 3A, compare lanes 5 and 6) and lacked phosphopeptides a’ and b’ (Fig. 3B, panel 4). Moreover, the tryptic digest of mRARy2S66A/S68A lacked phosphopeptides a’ and b’ (Fig. 3B, panel 5), indicating that mRARy2 is phosphorylated in its A/B region at serines 66/68.

Deletion of the F region in mRARy2ΔF decreased the overall phosphorylation level (Fig. 3A, lane 8), and phosphopeptides d and e disappeared (Fig. 3B, panel 6). Moreover, a chimeric receptor construct expressing the DEF regions of mRARy fused
to the DNA binding domain of the yeast transactivator Gal4 [Gal4-mRARγ(DEF)] was also phosphorylated when overexpressed in COS-1 cells (Fig. 3A, lane 11) and yielded phosphopeptides d and e (Fig. 3B, panel 3). When the region F serine 440, which belongs to a potential phosphorylation site for proline-dependent kinases (Fig. 1), was mutated to alanine, the corresponding mutant construct, mRARγS440A, did not yield the two phosphopeptides d and e (data not shown), which, as mentioned above, may correspond to partial trypsin digestion products (37). Thus, mouse RARγ contains a phosphorylation site in its F region, that is absent in human RARγ.

Mouse RARγ2 is the main RARγ isoform present in F9 embryonal carcinoma cells (21, 23). This endogenous RARγ isoform was also phosphorylated (Fig. 3C) in its B and F regions, as it yielded an array of tryptic phosphopeptides similar to that observed with recombinant mRARγ2 overexpressed in COS-1 cells (compare Fig. 3D with Fig. 3B, panel 2).

RARγ Is Phosphorylated in Vitro by cdk7 Present in the CAK Complex and TFIIH—We then investigated whether like RARα (14), RARγ could be phosphorylated by cdk7 contained in either CAK (cdk7/cyclin H-MAT1 complex) or the general transcription repair factor TFIIH (17). Purified bacterially expressed rat CAK was phosphorylated in vitro by CAK either free or as a component of TFIIH (Fig. 4A, lane 4–6). Interestingly, hRARγ1 was more efficiently phosphorylated by TFIIH than by CAK (Fig. 4A, compare lanes 2 and 3, autoradiography and Western blot), irrespective of the presence of RA (data not shown).
hRARγ phosphorylated in vitro by either CAK (Fig. 4C, panel 1) or TFIIH (data not shown) yielded the same phosphopeptide pattern as hRARγ overexpressed in COS-1 cells. hRARγS77A was less phosphorylated (Fig. 4B, lane 2) and lacked phosphopeptide a (Fig. 4C, panel 2). Both hRARγS79A and hRARγS77A/S79A were not phosphorylated (Fig. 4B, lane 3 and data not shown) and migrated as single species corresponding to the faster migrating non-phosphorylated form of hRARγ. Altogether, these data indicate that hRARγ is a substrate for cdk7 within CAK and TFIIH.

Because serines 77 and 79 belong to consensus motifs for proline-directed kinases (38–40), we also investigated whether RARγ could be a substrate for mitogen-activated protein kinases. Although hRARγ was phosphorylated by p44MAPK (Fig. 4B, lane 4), tryptic phosphopeptide mapping yielded only phosphopeptide a (Fig. 4C, panel 3). Only the S77A mutation and not the S79A one abrogated this RARγ phosphorylation (Fig. 4B, compare lanes 4 to 7). Thus, although RARγ can be a substrate for MAPK in vitro, phosphorylation by this kinase is different from that achieved with cdk7.

RARγ and RARα Bind Both to cdk7 and to Several Subunits of the Core of TFIIH—As previously reported in the case of RARα (14), RARγ directly interacted with cdk7. Purified bacterially expressed hRARγ was indeed bound by recombinant cdk7 immunoadsorbed onto protein A-Sepharose beads cross-linked with cdk7 antibodies (Fig. 5, lane 2). Similarly to RARαAB (14), RARγAB was also bound by cdk7 (Fig. 5, lane 3), indicating that cdk7 does not bind to the A/B region.

The observation that RARγ was more efficiently phosphorylated by cdk7 within TFIIH than within CAK prompted us to investigate whether this resulted from a tighter binding of RARγ to TFIIH than to free CAK. TFIIH is a multisubunit complex composed of CAK and of six additional subunits (p34, p44, p52, p62, XBP, and XPD) referred to as the core of TFIIH (17). Sf9 cells were therefore cotransfected with baculoviruses expressing RARγAB and either the whole TFIIH (nine subunits, IIH9) or some TFIIH subcomplexes, such as the “core TFIIH/XPD” (six subunits, IIH6), the core TFIIH (IIH5), or CAK (17). Infected cell extracts were then immunoprecipitated with monoclonal antibodies directed against either the p44 subunit of TFIIH or RARγ. The immunoprecipitated fractions (mentioned as “Bound (B)” in Fig. 6) were resolved by SDS/PAGE and analyzed by immunoblotting using antibodies against either XBP and p62 subunits of TFIIH or cdk7, cyclin H, and MAT1 subunits of CAK. mAb-p44 retained the corresponding IIH complexes (Fig. 6A, lanes 1–8) and also RARγ (Fig. 6A, lanes 4, 6, and 8). Conversely, mAb-RARα retained not only RARγ but also the TFIIH subcomplexes, whether the core of TFIIH was produced or not in association with CAK (Fig. 6B, lanes 2 and 6), indicating that RARγ can bind not only CAK but also the core of TFIIH. RARγ also communoprecipitated with cdk7, cyclin H, and MAT1 when cotransfected with CAK only (Fig. 6B, lane 10).

Similarly, when Sf9 cells were cotransfected with FLAG-tagged RARα and either IIH9, IIH6, or IIH5, mAb-p44 communopre-
Reciprocally, monoclonal anti-FLAG antibodies coimmunoprecipitated RARγ2 with cdk7 and TFIIH in F9 cells extracts. A, nuclear extracts from WT and RARγ2−/− F9 cells (1 mg) were immunoprecipitated (IP) with mAb-p44 (lanes 3 and 4) and immunoprobed with either mAb-p62, mAb-cdk7, or RPγ(F). Lanes 1 and 2 correspond to aliquots of the unimmunoprecipitated extracts. B, nuclear extracts from WT and RARγ2−/− F9 cells were immunoprecipitated with either mAb5D2 (lanes 5 and 6) or mAb-glutathione S-transferase (lanes 3 and 4) and probed with RPγ(F) and mAb-cdk7. Aliquots of unimmunoprecipitated extracts are shown in lanes 1 and 2.

Collectively, these results show that RARγ and RARα interact with TFIIH through both Cdk7 and the TFIIH core. Moreover, a direct interaction between RARα (Fig. 6E) and RARγ (data not shown) and either one of the core TFIIH subunits, XPB, XPD, p52 (Fig. 6E, lanes 2, 6, and 14), p44, or p34 (data not shown), could be revealed, indicating that these subunits may interact with RARs within TFIIH. Note that no interaction was observed between p62 and RARα, whereas the immunoprecipitations were carried out with mAb-p62 or mAb-RAR (Fig. 6E, lane 10, and data not shown).

To investigate whether RARγ was also intracellularly associated with TFIIH, we used the murine F9 cell line in which mRARγ2 is phosphorylated (see above). Nuclear extracts from F9 cells were immunoprecipitated with monoclonal antibodies against p44 and analyzed by SDS-PAGE/immunoblotting using antibodies against either p62, cdk7, or RARγ. mAb-p44 coimmunoprecipitated TFIIH and RARγ (Fig. 7A, lane 3). Reciprocally, mAb-RARγ coimmunoprecipitated RARγ and cdk7 (Fig. 7B, lane 5). These interactions were specific, as they were not revealed in control immunoprecipitations performed with mAb against the unrelated glutathione S-transferase protein (Fig. 7B, lanes 3 and 4) or with nuclear extracts from RARγ null F9 cells (RARγ−/− F9 cells) (Fig. 7B, lanes 2 and 6 and Fig. 7A, lane 4). Similar results were observed with RARα in the same cells (data not shown). Altogether these data indicate that a fraction of RARγ2 and RARα is associated with TFIIH in F9 cells.

Phosphorylation by cdk7 Modulates the Transcriptional Activity of RARγ—To investigate whether phosphorylation of the A/B region of RARγ could play a role in the ligand-independent AF-1 transactivation function, we first tested the activity of hRARγ1(AB)-ER(C) (a fusion of the A/B region of hRARγ1 with the ER DNA binding domain, see above) in transient transfection assays using the CAT reporter construct, mCRBPII(17m-ERE)-CAT (8). hRARγ1(AB)-ER(C) activated 3-fold the expression of the reporter, whereas mutation of serines 77 and 79 into alanine abrogated this stimulation, indicating the importance of these serines for AF-1 activity (Fig. 8).

The role of phosphorylation of the AF-1 domain on the transactivation properties of RARγ was further investigated using the full-length receptor and a reporter construct containing the CAT gene under the control of a RA-inducible promoter, the natural mRARβ2 promoter (7). Transcription from this promoter was stimulated by hRARγ1WT in the presence of a selective ligand (the RARγ agonist, BMS98161 at 10−7 M (Fig. 9A)). Deletion of the A/B region abrogated transcriptional activation, in agreement with previous reports (7). Mutation into alanine of Ser-77 and Ser-79 located in the A/B region reduced the transcriptional activity of hRARγ1 (Fig. 9A), confirming that these phosphorylation sites are required for optimal transcription. That phosphorylation of serines 77 and 79 by cdk7 could be responsible for efficient transcription was further supported by the observation that overexpressed cdk7 significantly enhanced transcription by hRARγ1WT but not by hRARγ1S77A/S79A (Fig. 9B). This increase did not occur with cdk7 devoid of kinase activity through mutation within its ATP binding site (cdk7m) (Fig. 9C). Importantly, overexpression of other proline-dependent kinases such as cdk1 or p44MAPK did not enhance the transcriptional activity of hRARγ1 (data not shown).

Because the phosphorylation sites located in the B region are the same in RARγ1 and RARγ2 isoforms and are conserved between human and mouse (see above and Fig. 1), similar results were expected with mRARγ2. In fact, transactivation by mRARγ2 was also reduced by mutation of serines 66 and 68 into alanine (Fig. 9D) and overexpressed cdk7-enhanced tran-
of two independent experiments.

Expression vectors were cotransfected with the mRAR

b961 at 10⁻² M for 20 h. The results are the mean of three independent experiments. B, increasing concentrations of hRAR1WT or S77A/S79A expression vectors were cotransfected with the mRARβ-CAT reporter as in A in the presence or absence of the cdk7 vector (0.5 μg). The results correspond to a representative experiment among three. C, COS-1 cells were cotransfected as in A without (lane 1) or with hRAR1WT expression vector (0.2 μg) in the absence (lane 2) or presence of the cdk7 wt (lane 3) or mutated (cdkm, lane 4) expression vectors. The results are the mean of two independent experiments. D, the mRARβ-CAT reporter gene was cotransfected as in A without or with increasing amounts of mRAR2WT, ΔAB, S66A/S68A, ΔF, and S440A expression vectors. The results correspond to a representative experiment among three. E, cotransfections were performed as in B with increasing concentrations of mRAR2WT and S66A/S68A expression vectors and in the presence or absence of the cdk7 vector. The results correspond to a representative experiment among three.

FIG. 9. Transactivation of the mRARβ2 promoter by RARγ is reduced by mutation of the phosphorylation sites located in the B region and is increased by overexpressed cdk7. A, COS-1 cells were cotransfected with the mRARβ2-CAT (5 μg) reporter gene without or with increasing amounts (0.1, 0.2, and 0.5 μg) of hRAR1 WT, S77A/S79A, or ΔAB expression vectors and treated with a RARγ-specific ligand (BMS 961) at 10⁻² M for 20 h. The results are the mean of three independent experiments. B, increasing concentrations of hRAR1WT or S77A/S79A expression vectors were cotransfected with the mRARβ2-CAT reporter as in A in the presence or absence of the cdk7 vector (0.5 μg). The results correspond to a representative experiment among three. C, COS-1 cells were cotransfected as in A without (lane 1) or with hRAR1WT expression vector (0.2 μg) in the absence (lane 2) or presence of the cdk7 wt (lane 3) or mutated (cdkm, lane 4) expression vectors. The results are the mean of two independent experiments. D, the mRARβ2-CAT reporter gene was cotransfected as in A without or with increasing amounts of mRAR2WT, ΔAB, S66A/S68A, ΔF, and S440A expression vectors. The results correspond to a representative experiment among three. E, cotransfections were performed as in B with increasing concentrations of mRAR2WT and S66A/S68A expression vectors and in the presence or absence of the cdk7 vector. The results correspond to a representative experiment among three.

Discussion

In this study, we have demonstrated that RARγ is phosphorylated in the N-terminal region that contains the activation function AF-1 (8) and plays an essential role in RA-induced primitive endodermal differentiation of F9 cells (13). This phosphorylation, which involves two serine residues, is ligand-independent and appears to be most efficiently performed by the cyclin H-dependent kinase cdk7, a component of the general transcription/DNA repair factor TFIIH (17, 41, 42). Interestingly, this phosphorylation modulates the activity of AF-1 in a responsive gene-dependent manner.

RARγ Is Phosphorylated by cdk7 in Its B and F Regions—
RARγ is phosphorylated at two phosphorylation sites located in the B region. These sites are present in both the γ1 and γ2 isoforms and are conserved between human and mouse (21, 23). They have been identified to serines 66 and 68 in RARγ2 and to serines 77 and 79 in RARγ1 (see Fig. 1). In this latter case, our data show that phosphorylation of serine 77 depends on that of serine 79. As previously reported for RARα (14), we demonstrate that these sites are phosphorylated by cdk7, a cyclin H- and MAT1-dependent kinase. Indeed, overexpression of wild type cdk7, but not of cdk7 mutated at its ATP binding site or of any other cdk, results in a higher level of phosphorylation of the various RARs so far tested (Ref. 14). Moreover, similarly to RARα, the pattern of phosphorylation of RARγ appears to be independent of the phases of the cell cycle (Ref. 14).3

As previously reported for RARα, RARγ is also phosphorylated in its F region. However, this phosphorylation concerns only mouse RARγ (either the γ1 or the γ2 isoforms) and not its human counterpart, due to the lack of conservation of this region between human and mouse (see Fig. 1). In contrast to phosphorylation of region B, no role has yet been found for this F region phosphorylation, either in RARγ or in RARδ.

RARγ Is More Efficiently Phosphorylated by TFIIH than by Free CAK—Cdk7 is associated with cyclin H and MAT1 in the CAK complex, and in the cell CAK is found either free or complexed with TFIIH, a general transcription factor also involved in DNA repair (17–20). Interestingly, we have shown that RARγ is more efficiently phosphorylated in vitro by cdk7 when included in TFIIH rather than in CAK, as previously reported for RARα (14) and for the CTD of RNA polymerase II (19, 41, 43, 44). That RARs are more efficiently phosphorylated by TFIIH than by CAK may result from RAR interactions not only with cdk7, as previously reported (Ref. 14 and Fig. 5), but also with core subunits of TFIIH. Indeed, in coimmunoprecipitations experiments performed with insect cells coinfected with baculoviruses expressing different subunits of TFIIH and either RARγ or RARα, we found that both RARs interact not only with cdk7 in CAK and TFIIH but also with several subunits of the TFIIH core. Thus, these multiple interactions may account for more efficient phosphorylation by cdk7 within TFIIH than within free CAK. Note that other transcription factors such as p53 and E2F-1, which have been shown to be phosphorylated by cdk7, also interact with the core subunits of TFIIH (45–47).

How cdk7 and the different TFIIH subunits interact with RARs remains to be investigated, but it is already clear from this and previous (14) studies that the N-terminal A/B region is not mandatory for these interactions. Moreover, the interaction of TFIIH with RARs is not sensitive to deletion of the AF-2AD core/helix 12,3 which is involved in the coactivator binding surface of the ligand binding domain (1). This is in accordance with our observation that the interaction of RARs with cdk7 and TFIIH is ligand-independent. Moreover, it suggests that the interaction between RARs and TFIIH involves another surface and is therefore mechanistically distinct from that described between RARs and coactivators (see the Introduction).

Regulation of Transcription by RARγ through Phosphorylation of the AF-1 Domain by TFIIH-associated cdk7—To investigate whether phosphorylation of the B region of RARs could modulate the ligand-induced activation of transcription, two reporter genes under the control of different responsive elements and promoters were tested: the natural mRARβ2 promoter, which contains a RARE with directly repeated motifs separated by 5 nucleotides (DR5), and the synthetic (TRE3)3tk promoter, which contains inverted (palindromic) repeated motifs. In both cases, RARγ activated transcription upon ligand binding. However, mutation of the phosphorylation sites located in the A/B region reduced transcription from the mRARβ2 promoter-based reporter gene, whereas it enhanced that from the (TRE3)3tk promoter-based reporter gene. The three-dimensional conformation of bound RXR/RAR heterodimers is most likely different on the two types of response elements. This may result in distinct steric conformations of the AF-1-activating domain and, therefore, in different interactions with putative AF-1 coactivators, which could be differentially modulated by phosphorylation. In this respect we note that interactions between coactivators and the AF-1-activating domain of either the estrogen receptor ERβ or the nuclear receptor SF-1 have been recently shown to be modulated by AF-1 phosphorylation (48, 49). In any event, such a possibility is in accordance with our previous report showing that phosphorylation of RARγ AF-1 is differentially required for RA-induced expression of target genes in F9 cells (13). Additionally, RAR phosphorylation may modulate the activity of TFIIH-associated cdk7 and/or regulate the enzymatic activity of some TFIIH subunits such as XBP and XPD that possess ATPase and helicase activity (18, 50–54) and are involved in distinct transcriptional steps (17).

In conclusion, we have demonstrated that, as other transcriptional regulators such as p53 and E2-F (45, 46, 55), RARs are targets for phosphorylation by cdk7 and interact with TFIIH. Thus, phosphorylation by TFIIH may be a general way of modulating the activity of transcriptional regulators. However, our present data also show that another proline-dependent kinase, p44MAPK, can also phosphorylate RARγ in vitro. Even though phosphorylation by this kinase is different from that achieved with cdk7 and overexpression of MAPK in COS cells does not affect the phosphorylation level and the transcription properties of RARγ, our present data do not rule out the possibility that p44MAPK could modulate RARγ activity in other cell types upon activation of the growth factor/MAPK cascade, as previously reported for ERO (56).

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