Ligand-induced gene activation by nuclear receptors (NRs) is a complex process requiring dissociation of corepressors and recruitment of coactivators. The putative transcriptional intermediary factor TIF1α has been previously characterized as a nuclear protein that interacts directly with the AF-2 ligand-dependent activating domain present in the ligand-binding domain of numerous steroid and nonsteroid receptors, including the estrogen (ERα) and retinoid X (RXRα) receptors. We report here that TIF1α is both a phosphoprotein and a protein kinase. TIF1α coexpressed in COS-1 cells with RXRα or ERα is phosphorylated and becomes hyperphosphorylated upon ligand treatment. This hyperphosphorylation requires the binding of TIF1α to transcriptionally active NRs since it is prevented by mutations either in the core (α-helix 12 of the ligand-binding domain) of the AF-2 activating domains of RXRα and ERα or in the NR box of TIF1α that are known to prevent TIF1α-NR interactions. Thus, TIF1α is a phosphoprotein that undergoes ligand-dependent hyperphosphorylation as a consequence of nuclear receptor binding. We further show that purified recombinant TIF1α possesses intrinsic kinase activity and that, in addition to auto-phosphorylation, TIF1α selectively phosphorylates the transcription factors TFIIeα, TAFii28, and TAFii55 in vitro. These latter results raise the possibility that TIF1α may act, at least in part, by phosphorylating and modifying the activity of components of the transcriptional machinery.

Nuclear receptors (NRs) represent a large family of ligand-inducible transcription factors that play numerous roles in the control of cell growth and differentiation, development, and homeostasis in response to small hydrophobic ligands, such as steroid and thyroid hormones, vitamin D, and retinoids (for reviews, see Refs. 1–4). Like other transcription factors, NRs display a modular structure with three main conserved regions: an N-terminal A/B region harboring an autonomous activation function (AF-1); a highly conserved C region encompassing most of the DNA-binding domain; and a C-terminal E region, which, in addition to a ligand-binding domain (LBD), contains a dimerization interface and a ligand-dependent activation function (AF-2). A well conserved amphipathic α-helix (helix 12) referred to as the AF-2 AD core has been identified in the C-terminal part of the E region and shown to be an essential element of the ligand-inducible AF-2 function (see Refs. 1–4). Upon ligand binding, this helix is thought to fold back over the LBD to generate transcriptionally active receptors (3, 5).

For controlling gene expression at the transcription level, NRs must interact with components required for the formation of stable preinitiation complexes, either directly or indirectly via transcriptional intermediary factors (mediators). While both types of interactions have been found for a number of NRs (for reviews, see Refs. 3 and 6), direct interactions are not sufficient to account for transcriptional interference/squelching between receptors (7–9) and for the role of receptor-associated cofactors that have been recently identified on the basis of their ability to interact with receptors in an agonist- and AF-2 AD core-dependent manner. These putative mediator proteins include TIF1α, RIP-140, SUG1, SRC-1 (and the related proteins TIF2/GRIP1, pCIP/ACTR/AIB1/RAC3), and CBP/p300 (3, 10–13). Some of these putative coactivators (e.g. SRC-1, TIF2/GRIP1, pCIP/ACTR/AIB1/RAC3, and CBP) contain an intrinsic activation function and can both relieve NR-induced squelching and stimulate the AF-2 activity of the NRs with which they interact (3, 10–13). Although CBP has been recently shown to be associated with RNA polymerase II via RNA helicase A (14), the molecular mechanisms by which cofactors function have not yet been identified. That enzymatic activities may be involved is strongly suggested by the recent findings that SUG1 is a DNA helicase (15), whereas SRC-1, pCIP/ACTR/AIB1/RAC3, and CBP/p300 possess histone acetyltransferase activities in vitro (16–20) and p300 can, in addition, acetylate basal transcription factors such as TFIIeβ and TFII F21.

TIF1α belongs to a new family of nuclear proteins that also

...retinoid X receptor; E2, estradiol; 9-cis-retinoic acid; OHT, 4-hydroxytamoxifen; PIPES, 1,4-piperazinediethanesulfonic acid; NEB, nuclear extraction buffer; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; TIF, transcriptional intermediary factor; TBP, TATA-binding protein; TAF, TBP-associated protein; GST, glutathione S-transferase.
includes TIF1α and TIF1γ (22). Based on amino acid homologies, a typical TIF1 domain structure has been ascribed to members of this family, comprising an N-terminal region that contains a RBCC (RING-finger-B boxes-coiled coil) motif, a poorly conserved central region, and a C-terminal region that contains a PHD finger and a bromodomain (22, 23). TIF1α was originally identified in a yeast genetic screen for mammalian proteins that can enhance the AF-2 activity of the retinoid X receptor (RXR) (23) and was subsequently found to interact with the AF-2 ADs of NRs in vivo as well as in vitro (23–25).

Additionally, TIF1α interacts with the heterochromatin-associated proteins HP1α, MOD1 (HP1β), and MOD2 (HP1γ) (22) and with the so-called transcriptional repression domain KRAB, which is present in about one-third of the vertebrate Kruppel-type (C2H2) zinc finger proteins (26, 27). These results raise the possibility that TIF1α may play a dual role in the control of transcription at the chromatin level, being involved both in repression through the formation of transcriptionally inactive heterochromatin and in hormone-dependent activation through reversion to transcriptionally active euchromatin (22, 27).

In this report, we demonstrate that TIF1α is a phosphoprotein that undergoes hyperphosphorylation upon interaction with liganded nuclear receptors in transfected cells. Moreover, TIF1α possesses intrinsic kinase activity responsible for auto-phosphorylation and phosphorylation of the transcription factors TFIIEα, TAFII28, and TAFII55 in vitro. These results suggest that hyperphosphorylation of TIF1α upon interaction with nuclear receptors, as well as phosphorylation of TFIIEα, TAFII28, and TAFII55 by TIF1α, might be instrumental in the control of transcription by nuclear receptors.

EXPERIMENTAL PROCEDURES

Plasmids—All plasmids used in transfection studies in COS-1 cells were pBGS (28) derivatives already described (22). The His-TIF1α construct described by Le Douarin et al. (23) was modified by replacing the BamHI/XhoI fragment of the pAc5HSigNT-B baculovirus expression vector (PharMingen) with the BglII/XhoI fragment of pET15b, thus eliminating the protein kinase A site, but preserving the reading frame and the His8 tag.

Cells, Transfections, and Nuclear Extract Preparation—COS-1 cells grown in Dulbecco’s modified Eagle’s medium containing 5% fetal calf serum were transfected using the calcium phosphate technique and grown in Dulbecco’s modified Eagle’s medium containing 5% fetal calf serum with 90 μg/ml of antibiotic gentamicin (Gibco BRL). The cells were harvested, washed in phosphate-buffered saline, and lysed in nuclear isolation buffer (5 mM PIPES, pH 7.5, 0.2 mM dithiothreitol, 10% glycerol, 0.5 mM phenyl-

RESULTS AND DISCUSSION

Ligand- and Nuclear Receptor-dependent Modification of TIF1α by Phosphorylation—The observation that TIF1α interacts with liganded NRs in vitro, but does not stimulate the AF-2 activity of NRs in transfected cultured cells (23), prompted us to examine the mechanism through which TIF1α might act. COS-1 cells were transiently transfected with TIF1α in the presence or absence of RXRα or ERO and their respective ligands (10−7 M 9C-R and E2, respectively). The nuclear ex-
correspond to nuclear extracts from COS-1 cells cotransfected with TIF1α (mAb4T). A single immunoreactive species with a molecular agreement with the expected molecular mass of the TIF1α presence of E2 (107; and RXRα (23), could be phosphorylated. The above COS-1 cells nuclear extracts were prepared, resolved by SDS-PAGE, and immunoblotted with the TIF1α-specific monoclonal antibody (mAb4T) that does not cross-react with simian TIF1α in COS cells. The positions of TIF1α and of the hyperphosphorylated form of TIF1α (TIF1αP) are indicated by arrows. B, COS-1 cells were cotransfected with TIF1α and RXRα expression vectors (lanes 1–4) and treated (lanes 2–5) or not (lane 1) with 9C-RA (10−7 M). Nuclear extracts were prepared and incubated with calf intestinal alkaline phosphatase (CIP; 20 units) with and (lane 4) or without (lane 3) the phosphatase inhibitor sodium vanadate (VAN; 10 m) and immunoblotted as described for A. Lane 5 corresponds to nuclear extracts from COS-1 cells cotransfected with the mutant TIF1α L730A/L731A and RXRα and treated with 9C-RA (10−7 m). C, nuclear extracts from COS-1 cells cotransfected with TIF1α and ERα expression vectors (lanes 1–4) in the absence (lane 1) or presence (lanes 2–4) of E2 (10−7 m) were treated with calf intestinal alkaline phosphatase with (lane 4) or without (lane 3) phosphate inhibitor vanadate as described for A. Lanes 5 and 6 correspond to nuclear extracts from COS-1 cells cotransfected with TIF1α and the AF-2 AD core ERα mutant M543A/L544A in the absence or presence of E2 (10−7 m), respectively. D, COS-1 cells cotransfected with TIF1α and RXRα expression vectors were labeled with [32P]orthophosphate in the presence or absence of 9C-RA (10−7 m). Extracts were immunoprecipitated with mAb4T and resolved by SDS-PAGE followed by autoradiography (lanes 1–4) and Western blotting with mAb4T (WB; lanes 5–8). Lanes 1 and 2 show the level of phosphorylation of TIF1α in the absence of RXRα. Cotransfection of RXRα resulted in a moderate increase in the radioactivity of the immunoreactive TIF1α band (lanes 3 and 7; presumably due to the presence of low amounts of 9C-RA in the cell culture medium), which was markedly enhanced by addition of 10−7 m 9C-RA (lanes 4 and 8). A nonspecific band (ns), which was previously undetected in A and B, is visible on Western blots, probably resulting from an enrichment by immunoprecipitation and subsequent immunoblotting with the same mAb (lanes 5–8). Extracts immunoprecipitated with TIF1α and RXRα expression vectors (lanes 1–4) in the absence (lane 1) or presence of 10−7 M E2 (lanes 2 and 4) and/or 10−7 M OHT (lanes 3 and 4). Immunoblotting analysis of the corresponding nuclear extracts indicated that OHT inhibited the E2 induction of the upper immunoreactive hyperphosphorylated TIF1α species (lane 4). F, COS-1 cells were transfected with TIF1α either alone (lanes 1 and 2) or in combination with wild-type RXRα (lanes 5 and 6) or RXRα mutated in the AF-2 AD core (RXRαA455–467) (lanes 7 and 8), RXRα M459A/L460A (lanes 9 and 10), and RXRα M459A/L460A/L461 (lanes 11 and 12). Cells were either untreated (lanes 1, 3, 5, 7, 9, and 11) or treated (lanes 2, 4, 6, 8, 10, and 12) with 10−7 m 9C-RA. Nuclear extracts were prepared and immunoblotted as described for A. Note that the RXRα AF-2 AD core mutants did not induce the appearance of the upper immunoreactive hyperphosphorylated TIF1α species. Lanes 3 and 4 correspond to cells transfected with RXRα only.

tracts from transfected cells were subjected to SDS-PAGE and immunoblotting with a TIF1α-specific monoclonal antibody (mAb4T). A single immunoreactive species with a molecular mass of ~120 kDa (Fig. 1A, lanes 1, 2, 5, and 6), which is in agreement with the expected molecular mass of the TIF1α protein (23), was detected in cells transfected with TIF1α alone in the absence or presence of ligand. Interestingly, a fraction of TIF1α showed a pronounced decrease in its electrophoretic mobility in ligand-treated cells cotransfected with either ERα (Fig. 1A, compare lanes 3 and 4) or RXRα (compare lanes 7 and 8). Thus, TIF1α could undergo a post-translational modification upon ligand-induced interaction with ERα or RXRα.

Since phosphorylation often alters protein mobility during SDS-PAGE, we examined whether TIF1α, a serine-rich protein (23), could be phosphorylated. The above COS-1 cells nuclear extracts were treated with calf intestinal alkaline phosphatase in the absence or presence of the phosphatase inhibitor sodium vanadate. Treatment with calf intestinal alkaline phosphatase alone abolished both the RXRα/9C-RA and ERα/E2-induced mobility shift of TIF1α (Fig. 1A, B, and C, compare lanes 1–3), whereas in the presence of vanadate, the slower migrating TIF1α species was unaffected (Fig. 1A, B, and C, lane 4). Thus, the interaction of TIF1α with the liganded nuclear receptors RXRα and ERα appears to promote phosphorylation of TIF1α.

To support this possibility, transfected COS-1 cells coexpressing TIF1α and RXRα were labeled with [32P]orthophosphate in the absence or presence of 9C-RA, and nuclear extracts were immunoprecipitated with mAb4T. Immunoprecipitates were subjected to SDS-PAGE, autoradiography, and immunoblotting (Fig. 1D). In the absence of RXRα and 9C-RA,
TIF1α was weakly phosphorylated (Fig. 1D, lanes 1, 2, 5, and 6). However, cotransfection of RXRα and treatment of the transfected cells with 9C-RA clearly induced hyperphosphorylation of TIF1α, resulting in a reduction of its electrophoretic mobility (Fig. 1D, lanes 4 and 8). Altogether, these results confirm that the liganded NR-induced modification occurs through phosphorylation, as it is abolished by phosphatase treatment and is accompanied by an increase in $^{32}$P labeling.

To investigate whether the hyperphosphorylation of TIF1α was specific for ligands that induce AF-2 activity, similar experiments were performed with ERα in the presence of the anti-estrogen OHT. OHT is known to prevent ERα AF-2 activity in mammalian and yeast cells (34, 35) and to antagonize the E₂-dependent interaction between the LBD of ERα and TIF1α (23). In COS cells cotransfected with TIF1α and ERα, OHT failed to induce any upward shift of TIF1α (Fig. 1E, compare lanes 1 and 3) and antagonized the shift induced by E₂ (compare lanes 2 and 4). This indicates that hyperphosphorylation of TIF1α requires the transcriptionally active conformation of the ERα holo-LBD that is required for interaction with TIF1α.

It has been previously demonstrated in vitro and in yeast (23) that the ligand-dependent interaction of NRs with TIF1α requires the integrity of the core of the AF-2 AD (α-helix 12). Therefore, we investigated whether this integrity was required for TIF1α hyperphosphorylation. Cotransfection experiments were performed with TIF1α and RXRα either lacking the AF-2 AD core (RXRαΔΔ555–467) or bearing amino acid substitution in the conserved hydrophobic residues (RXRαF455A/L456A and RXRαS459A/L460A) (23, 24, 36). In contrast to wild-type RXRα (Fig. 1F, lanes 5 and 6), the RXRα deletion mutant (lanes 7 and 8) and the AF-2 AD core point mutants (lanes 9–12) failed to induce the characteristic mobility shift seen upon hyperphosphorylation. Similar amino acid mutations in the AF-2 AD core of ERα (ERαM543A/L544A) that prevent the interaction with TIF1α (22) also eliminated the E₂/α-helix-induced modification of TIF1α (Fig. 1C, compare lanes 5 and 6 with lanes 1 and 2). These results, which are in agreement with the functional interactions detected both in yeast and in vitro (23, 24), demonstrate that TIF1α hyperphosphorylation induced by liganded RXRα or ERα requires the integrity of the AF-2 AD core (α-helix 12 of the LBD).

The ligand- and AF-2 AD core-dependent TIF1α-NR interaction is also known to require the TIF1α NR box (amino acids 726 to 735) (22), and point mutations in this NR box (TIF1αL730AL731A) abolish the binding of TIF1α to nuclear receptors (22). To determine whether the TIF1α NR box was required for NR-induced modification of TIF1α, wild-type and mutant (L730AL731A) TIF1α and RXRα expression vectors were cotransfected with RXRα in COS-1 cells with and without 9C-RA. Mutation of the NR box abolished TIF1α hyperphosphorylation (Fig. 1B, compare lanes 2 and 5). The same results were obtained when COS cells were cotransfected with NR box mutant TIF1α and ERα and treated with E₂ (data not shown). Thus, TIF1α hyperphosphorylation requires an intact NR box. Altogether, these results demonstrate that the phosphorylation of TIF1α, which occurs in the presence of liganded RXRα and ERα, exhibits the same requirements as those previously shown to be indispensable for the binding of TIF1α to the LBD of NRs.

**Autophosphorylation of Recombinant His-TIF1α.—** With the aim of identifying the kinase responsible for TIF1α phosphorylation, we produced recombinant His-TIF1α using a baculovirus expression vector in infected insect Sf9 cells and purified it to near homogeneity by DEAE-Sephadex followed by nickel chelate (Ni²⁺-nitrilotriacetic acid) affinity chromatography (see “Experimental Procedures”). After a series of high concentration salt washes, elution, and dialysis, purified TIF1α was tested in an in vitro kinase assay. TIF1α was phosphorylated without the addition of any other factors or kinase, suggesting that TIF1α might have an autokinase activity (Fig. 2A, lanes 4 and 11).

To eliminate the possibility that this kinase activity might be due to a copurifying contaminating kinase, an additional immunopurification step was performed. Purified His-TIF1α was immunopurificated using three different monoclonal antibodies (mAb1T, mAb2T, and mAb7T), each recognizing a different TIF1α epitope. His-TIF1α bound to the antibodies was washed extensively with 1 M KCl before elution with an excess of the cognate epitope peptide. The remaining supernatant (Fig. 2A, Sn), the peptide eluate (E) containing TIF1α, and the eluted beads (Bd) were subjected to an in vitro kinase reaction and analyzed by SDS-PAGE and autoradiography. Both the immunopurification and elution steps were efficient (Fig. 2A, lanes 1, 2, 5, 6, 8, and 9). Strikingly, TIF1α eluted from the three immunoprecipitates and incubated in the kinase reaction was phosphorylated (Fig. 2A, lanes 3, 7, and 10). Thus, after three purification steps (DEAE-Sephadex, nickel chelate, and immunoaffinity chromatography), His-TIF1α retained the ability to autophosphorylate.

![Fig. 2. Recombinant His-TIF1α is an autokinase.](image-url)
To eliminate the possibility that a copurifying kinase remained associated with TIF1α after these three purification steps, His-TIF1α eluted from mAb1T was resolved by SDS-PAGE and silver-stained. No contaminating polypeptides could be detected (Fig. 2B). The resolved His-TIF1α was tested for autokinase activity in an in-gel kinase assay. Only one protein corresponding in size to His-TIF1α was phosphorylated (Fig. 2C), thus ruling out the possibility that the phosphorylation of TIF1α could be due to a contaminating kinase. No signal was revealed in controls performed with uninfected or wild-type baculovirus-infected Sf9 cells (data not shown), thus corroborating the specificity of our results.

TIF1α does not possess any obvious well conserved domains typical of serine kinases. However, the Rossmann kinase ATP-binding motif GXGXXG, might be loosely conserved at amino acids 659–669 (GSRGSSGSSSK) of the TIF1α amino acid sequence (23). As TIF1α is very rich in serine and threonine residues (23), further mutational analysis will be required to define the phosphorylated amino acids and to identify the kinase ATP-binding motif.

TIF1α Phosphorylates Certain Transcription Factors in Vitro—We next investigated whether TIF1α could phosphorylate substrates other than itself. The first proteins to be tested were those known to interact directly with TIF1α, such as RXRα, ERα, HP1α, and MOD1 (HP1β) (22, 23). None of the corresponding recombinant proteins were phosphorylated by purified TIF1α in an in vitro kinase assay (data not shown). To investigate whether TIF1α could phosphorylate ERα and RXRα in a cellular environment, COS-1 cells cotransfected with TIF1α and either ERα or RXRα were labeled with [32P]orthophosphate and, after disruption, were immunoprecipitated with ER or RXR antibodies. The phosphorylation of ERα was induced by estradiol (Fig. 3A, compare lanes 5 and 6). However, this phosphorylation was not affected by overexpression of either wild-type TIF1α (lanes 1 and 2) or TIF1α mutated in the NR box (lanes 3 and 4). Similarly, TIF1α did not affect the level of phosphorylation of RXRα, even in the presence of 9C-RA (Fig. 3B).

TIF1α is a putative transcriptional intermediary factor for nuclear receptors (22, 23). Therefore, the ability of TIF1α to phosphorylate recombinant transcription initiation factors such as TFIIFαβ, TFIIFαγ, TFIIEα, TFIIEβ, TBP, TAFII135, TAFII20, TAFII18, TAFII28, and TAFII55 (31, 32) was investigated in vitro (Fig. 4A) (data not shown). Among these factors, only TFIIEα and the TBP-associated factors TAFII28 and TAFII55 were phosphorylated by immunopurified recombinant His-TIF1α (Fig. 4A, lanes 6, 12, and 14). However, no stable interaction of TIF1α with these factors could be found (data not shown), as previously observed with other known components of the general transcriptional machinery (23).

Conclusion—TIF1α was originally identified as a transcriptional intermediary factor on the basis of its direct ligand-dependent interaction with NRs in vitro and in vivo (23, 24). Results from our laboratory have provided evidence supporting the idea that TIF1α may control, at least in part, transcription by NRs through chromatin remodeling (22). We demonstrate here that TIF1α undergoes hyperphosphorylation that is dependent on direct interaction with a nuclear receptor upon binding of the cognate ligand. We also demonstrate that TIF1α possesses an intrinsic kinase activity as it phosphorylates and phosphorylates transcription factors such as TFIIEα, TAFII28, and TAFII55 in vitro. Thus, TIF1α is both a phosphoprotein and a protein kinase.

TIF1α contains several conserved domains, including a bromodomain that is also present in a number of transcriptional regulatory proteins (22, 23, 37). In most members of the bromodomain family, this domain was found in association with other conserved domains, some of which possess an enzymatic activity (helicase, ATPase, or histone acetyltransferase activity) (37). Interestingly, the 290-kDa TATA-binding protein-associated factor of RNA polymerase II, TAFII250, which contains a bromodomain, has been shown to possess a histone acetyltransferase activity and two kinase domains (38), whereas the Brahma proteins (BRM or SNF2H) and the TBP-associated factors TAFII250, which contains a bromodomain, have been shown to possess a histone acetyltransferase activity and two kinase domains (38), whereas the Brahma proteins (BRM or SNF2H) and the TBP-associated factors TAFII250, which contains a bromodomain, have been shown to possess a histone acetyltransferase activity and two kinase domains (38), whereas the Brahma proteins (BRM or SNF2H) and the TBP-associated factors TAFII250, which contains a bromodomain, have been shown to possess a histone acetyltransferase activity and two kinase domains (38), whereas the Brahma proteins (BRM or SNF2H) and the TBP-associated factors TAFII250, which contains a bromodomain, have been shown to possess a histone acetyltransferase activity and two kinase domains (38), whereas the Brahma proteins (BRM or SNF2H) and the TBP-associated factors TAFII250, which contains a bromodomain, have been shown to possess a histone acetyltransferase activity and two kinase domains (38), whereas the Brahma proteins (BRM or SNF2H) and the TBP-associated factors TAFII250, which contains a bromodomain, have been shown to possess a histone acetyltransferase activity and two kinase domains (38). How the hyperphosphorylation of TIF1α, which is induced upon interaction with liganded nuclear receptors, might modulate its interaction with chromatin-associated proteins (22, 27) remains to be investigated. Further experiments are also required to study whether the activity of the factors associated with the general transcription machinery could be affected through phosphorylation by TIF1α.
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