The Putative Cofactor TIF1 Is a Protein Kinase That Is Hyperphosphorylated upon Interaction with Liganded Nuclear Receptors*

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 26, Issue of June 26, pp. 16199–16204, 1998

Printed in U.S.A.

The Putative Cofactor TIF1α Is a Protein Kinase That Is Hyperphosphorylated upon Interaction with Liganded Nuclear Receptors*

(Received for publication, March 4, 1998, and in revised form, April 13, 1998)

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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 autophosphorylation, TIF1α selectively phosphorylates the transcription factors TFIIEα, $TAF_{II}28$, and $TAF_{II}55$ *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) [1] 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 TFIIF (21).

TIF1α belongs to a new family of nuclear proteins that also

* This work was supported in part by CNRS, INSERM, the Hôpital Universitaire de Strasbourg, the Collège de France, the Association pour la Recherche sur le Cancer, the Fondation pour la Recherche Médicale, the Ligue Nationale contre le Cancer, and the Human Frontier Science Program. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‖ Supported by the Ministère de la Recherche et de l'Enseignement Supérieur.

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[1] The abbreviations used are: NRs, nuclear receptors; LBD, ligand-binding domain; AD, activating domain; TF, transcription factor; RXR, retinoid X receptor; E_2, estradiol; 9*C*-RA, 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 TIF1β and TIF1γ (22). [2] 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 Krüppel-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 autophosphorylation and phosphorylation of the transcription factors TFIIEα, $TAF_{II}28$, and $TAF_{II}55$ *in vitro*. These results suggest that hyperphosphorylation of TIF1α upon interaction with nuclear receptors, as well as phosphorylation of TFIIEα, $TAF_{II}28$, and $TAF_{II}55$ 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 pSG5 (28) derivatives already described (22). The His-TIF1α construct described by Le Douarin *et al.* (23) was modified by replacing the *Bam*HI/*Xho*I fragment of the pAcSGHisNT-B baculovirus expression vector (Pharmingen) with the *Bgl*II/*Xho*I fragment of pET15b, thus eliminating the protein kinase A site, but preserving the reading frame and the His_6 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 treated for 4 h with 10^{-7} M estradiol (E_2), 9*C*-RA, or 4-hydroxytamoxifen (OHT) when indicated. Cells were harvested, washed in phosphate-buffered saline, and lysed in nuclear isolation buffer (5 mM PIPES, pH 7.9, 2 mM $CaCl_2$, 85 mM KCl, and 5% sucrose) containing 0.3% Nonidet P-40 and protease inhibitor mixture. After centrifugation (5 min at 3000 rpm), nuclei were suspended in nuclear extraction buffer (NEB; 20 mM Tris-HCl, pH 7.5, 0.2 mM dithiothreitol, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture) containing 400 mM KCl. After four cycles of freezing and thawing and centrifugation (10 min at 13,000 rpm), the resulting nuclear extract was dialyzed against NEB containing 50 mM KCl.

Antibodies—Monoclonal (mAbs) anti-TIF1α antibodies were raised against synthetic peptides corresponding to amino acids 989–1005 (mAb1T), 762–776 (mAb2T), and 33–52 (mAb6T) and coupled to ovalbumin (23). Purified *Escherichia coli*-expressed proteins TIF1.22.2 (amino acids 682–1017) and TIF1.22.1 (amino acids 396–682) were also used as antigens and resulted in the production of mAb4T and mAb5T, respectively (23). Monoclonal antibodies against ERα (mAbB10) and RXRα (mAb4RX3A2) and polyclonal antibodies against RXRα (RPRXα(A)) were as described (29, 30).

SDS-PAGE and Western Blot Analysis—Nuclear extracts were subjected to SDS-8% PAGE and electrotransferred to nitrocellulose filters. The filters were incubated with mAb4T followed by a peroxidase-conjugated anti-mouse IgG secondary antibody and developed using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech).

Purification of Recombinant His-TIF1α—Plated Sf9 cells infected with baculovirus encoding a His-tagged TIF1α cDNA in a modified pAcSGHisNTB-B vector were collected in growth medium, pelleted (10 min at 3000 rpm and 4 °C), and resuspended in phosphate-buffered saline containing 30% glycerol. The cells were pelleted, homogenized in a Dounce homogenizer in NEB containing 400 mM KCl and protease inhibitor mixture, and centrifuged as described (15). The supernatant was dialyzed against load buffer (NEB containing 50 mM KCl) and loaded onto an equilibrated DEAE column, which was washed and eluted with load buffer containing 400 mM KCl. The eluate fraction was applied directly onto an equilibrated nickel chelate (Ni^{2+}-nitrilotriacetic acid) column (QIAGEN Inc.). The column was washed extensively with NEB containing 400 mM KCl and then 400 mM NaCl, followed by wash buffer (50 mM phosphate buffer, pH 6, containing 400 mM NaCl and 50 mM imidazole), and then eluted with 500 mM imidazole in the same buffer. The collected fractions were dialyzed against dialysis buffer (50 mM Tris-HCl, pH 7.9, 20% glycerol, and 0.5 mM dithiothreitol) containing 50 mM KCl, subjected to SDS-PAGE, and either Coomassie Blue- or silver nitrate-stained or analyzed by Western blotting as described above.

Immunopurification of the Ni^{2+}-nitrilotriacetic acid elution fractions was performed essentially as described (15). Briefly, the fraction was precleared with protein G-Sepharose for 1 h at 4 °C, and the supernatant was incubated with mAb bound to protein G-Sepharose in immunoprecipitation buffer (50 mM Tris-HCl, pH 7.9, 10% glycerol, 5 mM $MgCl_2$, and 0.5 mM dithiothreitol) containing 500 mM KCl. Bound protein was washed with a minimum of 50 bed volumes of immunoprecipitation buffer containing 1 M KCl before re-equilibration and peptide elution in immunoprecipitation buffer. Eluted fractions were dialyzed, analyzed by silver nitrate staining, and tested for kinase activity.

Expression and Purification of Transcription Factors—Expression in *E. coli* and purification of recombinant human TBP, His-TFIIAαβ, His-TFIIAγ, His-TFIIEα, and His-TFIIEβ were as described (31). His-tagged full-length $TAF_{II}28$ was overexpressed in *E. coli* and purified by Ni^{2+}-nitrilotriacetic acid affinity chromatography (32). GST-h$TAF_{II}55$ (where "h" is human) expressed in *E. coli* was immobilized on a glutathione-Sepharose column and digested with thrombin in the presence of 5 mM $CaCl_2$ in order to release full-length human $TAF_{II}55$. GST-h$TAF_{II}135$ (amino acids 372–1083), GST-h$TAF_{II}20$, and GST-h$TAF_{II}18$ (32) were purified by immobilization on glutathione-Sepharose and elution with reduced glutathione.

In Vitro and in Vivo Phosphorylation—*In vitro* phosphorylation reactions were performed with 5 ng of purified His-TIF1α in 20 mM HEPES, pH 7.9, 15 mM $MgCl_2$, 100 μM ATP, and 5 μCi of [γ-^{32}P]ATP with or without substrate (0.5 μg) as indicated. Reactions were performed at 30 °C for 30 min and stopped by the addition of Laemmli buffer and boiling. The phosphorylated proteins were subjected to 8% SDS-PAGE, Coomassie Blue-stained, and autoradiographed.

For *in vivo* phosphorylation, transfected COS-1 cells were starved overnight in phosphate-deficient medium and then incubated in the same medium for 4 h with 250 μCi/ml [^{32}P]orthophosphate as described (33). Whole cell or nuclear extracts were subjected to immunoprecipitation and SDS-PAGE and analyzed by autoradiography and immunoblotting (33).

In-gel Kinase Assay—Immunopurified His-TIF1α (50–500 ng) was subjected to 8% SDS-PAGE. After removing SDS from the gel by two washes in buffer A (50 mM Tris-HCl, pH 8, and 5 mM β-mercaptoethanol) containing 20% isopropyl alcohol, proteins were denatured in buffer A containing 6 M guanidine and gradually renatured by a series of five incubations over a 16-h period at room temperature in buffer A containing 0.04% Tween 40. The gel was equilibrated for 1 h in kinase buffer (40 mM HEPES, pH 7.6, 2 mM magnesium acetate, and 30 mM KCl), and the phosphorylation reaction was performed in the same buffer with 25 μCi of [γ-^{32}P]ATP for 1 h at room temperature. The gel was then extensively washed with 1% sodium pyrophosphate and 5% trichloroacetic acid, dried, and autoradiographed.

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 ERα and their respective ligands (10^{-7} M 9*C*-RA and E_2, respectively). The nuclear ex-

[2] L. Venturini, J. You, R. Galien, M. Stadler, V. Lallemand, M. H. M. Koken, M. G. Mattei, A. Ganser, P. Chamben, R. Losson, and H. de Thé, manuscript in preparation.

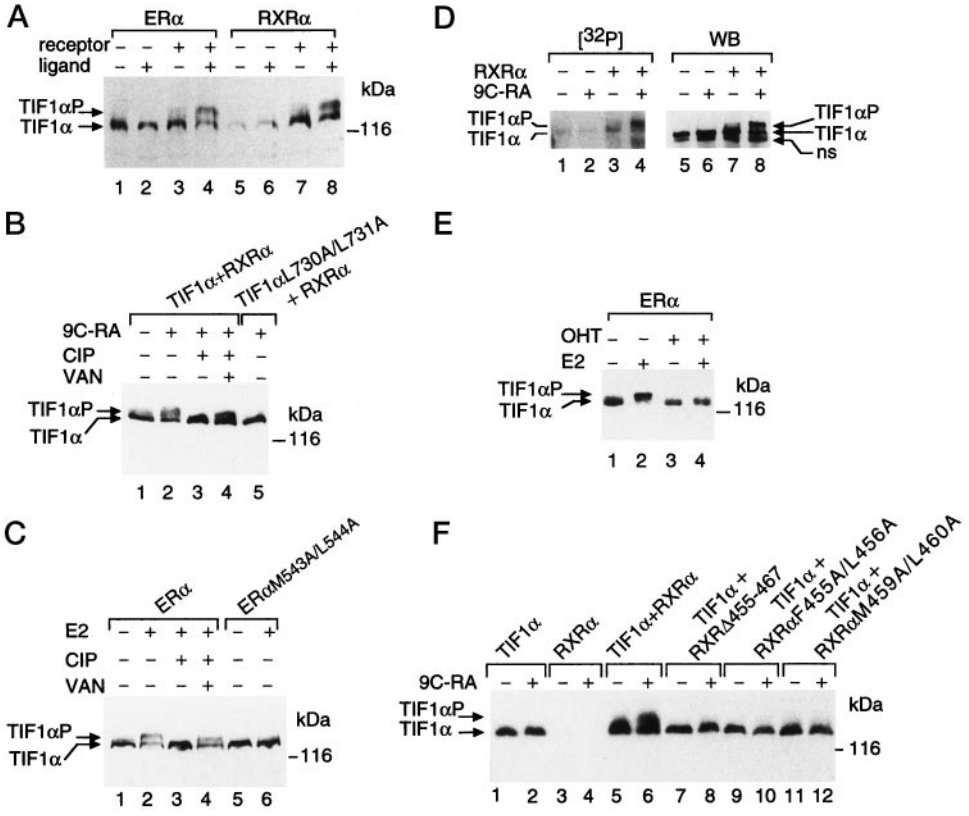

FIG. 1. **Ligand- and nuclear receptor-dependent phosphorylation of TIF1α.** *A*, COS-1 cells were transfected with mouse TIF1α expression vector (5 μg) either alone (*lanes 1*, *2*, *5,* and *6*) or in combination with human ERα (5 μg; *lanes 3* and *4*) or mouse RXRα (5 μg; *lanes 7* and *8*) expression vector in the absence (*lanes 1*, *3*, *5,* and *7*) or presence (*lanes 2*, *4*, *6*, and *8*) of the cognate ligand: E_2 or 9*C*-RA at 10^{-7} M. 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 9*C*-RA (10^{-7} M). Nuclear extracts were prepared and incubated with calf intestinal alkaline phosphatase (*CIP*; 20 units) and with (*lane 4*) or without (*lane 3*) the phosphatase inhibitor sodium vanadate (*VAN*; 10 nM) 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 9*C*-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 E_2 (10^{-7} M) were treated with calf intestinal alkaline phosphatase with (*lane 4*) or without (*lane 3*) the phosphatase inhibitor vanadate as described for *B*. *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 E_2 (10^{-7} M), respectively. *D*, COS-1 cells cotransfected with TIF1α and RXRα expression vectors were labeled with [^{32}P]orthophosphate in the presence or absence of 9*C*-RA (10^{-7} M). Extracts were immunoprecipitated with mAb4T and resolved by SDS-PAGE followed by autoradiography (*[^{32}P]*; *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 9*C*-RA in the cell culture medium), which was markedly enhanced by addition of 10^{-7} M 9*C*-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*). *E*, COS-1 cells were cotransfected with TIF1α and ERα expression vectors in the absence (*lane 1*) or presence of 10^{-7} M E_2 (*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 E_2 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 (RXRα-(Δ455–467) (*lanes 7* and *8*), RXRα F455A/L456A (*lanes 9* and *10*), and RXRα M459A/L460A (*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 9*C*-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/9*C*-RA- and ERα/E_2- induced mobility shift of TIF1α (Fig. 1, *B* and *C*, compare *lanes 1–3*), whereas in the presence of vanadate, the slower migrating TIF1α species was unaffected (Fig. 1, *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 [^{32}P]orthophosphate in the absence or presence of 9*C*-RA, and nuclear extracts were immunoprecipitated with mAb4T. Immunoprecipitates were subjected to SDS-PAGE, autoradiography, and immunoblotting (Fig. 1*D*). In the absence of RXRα and 9*C*-RA,

TIF1α was weakly phosphorylated (Fig. 1*D*, *lanes 1*, *2*, *5,* and *6*). However, cotransfection of RXRα and treatment of the transfected cells with 9*C*-RA clearly induced hyperphosphorylation of TIF1α, resulting in a reduction of its electrophoretic mobility (Fig. 1*D*, *lanes 4* and *8*). Altogether, these results confirm that the liganded NR-induced TIF1α 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 E_2-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 E_2 (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 (RXRα-(Δ455–467)) or bearing amino acid substitution in the conserved hydrophobic residues (RXRα F455A/L456A and RXRα M459A/L460A) (23, 24, 36). In contrast to wild-type RXRα (Fig. 1*F*, *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 (ERα M543A/L544A) that prevent the interaction with TIF1α (22) also eliminated the ERα/E_2-induced modification of TIF1α (Fig. 1*C*, 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 (TIF1α L730A/L731A) 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 (L730A/L731A) TIF1α and RXRα expression vectors were cotransfected with RXRα in COS-1 cells with and without 9*C*-RA. Mutation of the NR box abolished TIF1α hyperphosphorylation (Fig. 1*B*, 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 E_2 (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 (Ni^{2+}-nitrilotriacetic acid) affinity chromatography

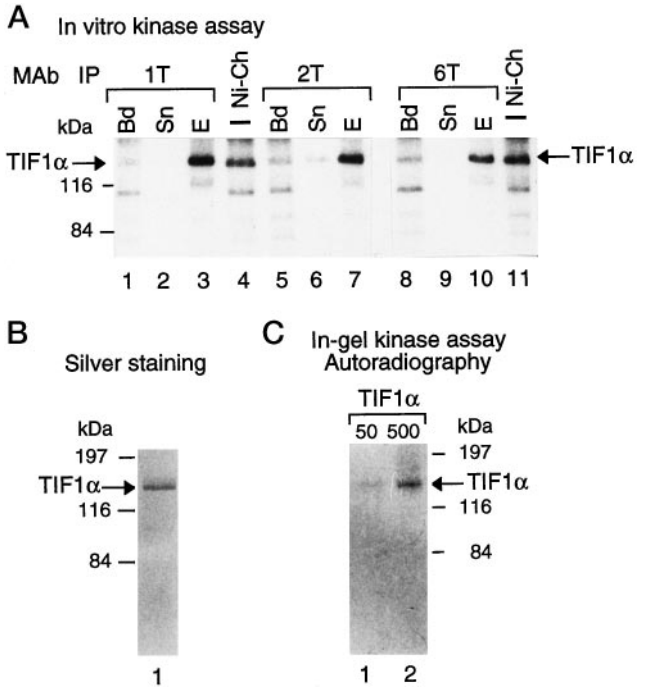

FIG. 2. **Recombinant His-TIF1α is an autokinase.** *A*, recombinant His-TIF1α expressed in insect Sf9 cells and purified by DEAE-Sephadex followed by nickel chelate affinity chromatography was immunoprecipitated with mAb1T (*lanes 1–3*), mAb2T (*lanes 5–7*), and mAb6T (*lanes 8–10*) as indicated. TIF1α was specifically eluted with the respective epitope peptides and dialyzed. The initial nickel chelate affinity chromatography fraction (*Ni-Ch; lanes 4* and *11*), the peptide eluate (*E; lanes 3*, *7,* and *10*), the remaining eluted beads (*Bd; lanes 1*, *5,* and *8*), and the supernatant (*Sn*) from the immunoprecipitates (*lanes 2*, *6,* and *9*) were each tested by an *in vitro* kinase assay. The phosphorylated proteins were resolved by SDS-PAGE and analyzed by autoradiography. The species detected at the top of the gel corresponds to TIF1α that was insufficiently reduced by the dithiothreitol present in the Laemmli buffer, as it disappeared upon addition of 5 mM β-mercaptoethanol (data not shown). *B*, the silver-stained gel of purified His-TIF1α eluted from mAb1T immunoprecipitates was subjected to SDS-PAGE. This affinity-purified fraction consists primarily of a single protein species migrating at the expected position of TIF1α. *C*, His-TIF1α eluted from mAb1T immunoprecipitates (~50 and 500 ng) was tested for autokinase activity in an in-gel kinase assay. The ^{32}P autoradiographic signal was coincidental with the position of the His-TIF1α protein.

(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. 2*A*, *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 immunoprecipitated using three different monoclonal antibodies (mAb1T, mAb2T, and mAb6T), 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. 2*A*, *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 immunoprecipitation and elution steps were efficient (Fig. 2*A*, *lanes 1*, *2*, *5*, *6*, *8,* and *9*). Strikingly, TIF1α eluted from the three immunoprecipitates and incubated in the kinase reaction was phosphorylated (Fig. 2*A*, *lanes 3*, *7,* and *10*). Thus, after three purification steps (DEAE-Sephadex, nickel chelate, and immunoaffinity chromatography), His-TIF1α retained the ability to autophosphorylate.

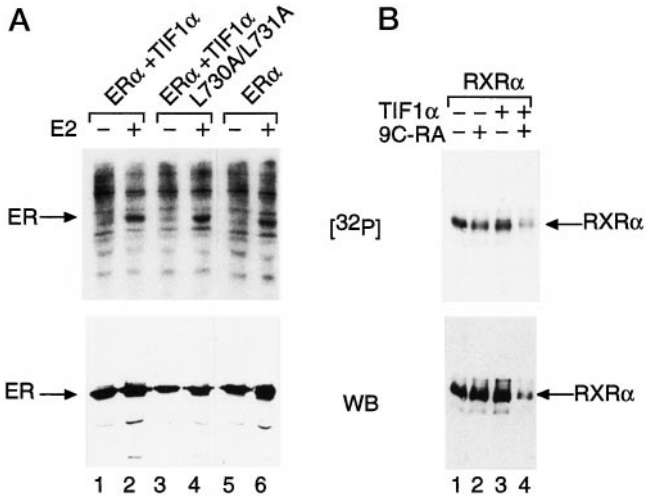

FIG. 3. **TIF1α does not affect the phosphorylation of ERα and RXRα.** *A*, COS-1 cells were transfected with ERα expression vector (5 μg) either alone (*lanes 5* and *6*) or in combination with TIF1α (*lanes 1* and *2*) or TIF1α L730A/L731A (*lanes 3* and *4*) expression vector (1 μg) in the absence (*lanes 1*, *3*, and *5*) or presence (*lanes 2*, *4,* and *6*) of E_2 (10^{-7} M). The cells were labeled with [^{32}P]orthophosphate. Cell extracts were immunoprecipitated with the ERα monoclonal antibody mAbB10 and resolved by SDS-PAGE followed by autoradiography (*[^{32}P]*) and Western blotting with mAbB10 (*WB*). *B*, COS cells were transfected with RXRα expression vector (5 μg) either alone (*lanes 1* and *2*) or in combination with TIF1α (1 μg) expression vector (*lanes 3* and *4*) in the absence (*lanes 1* and *3*) or presence (*lanes 2* and *4*) of 9*C*-RA (10^{-7} M). After [^{32}P]orthophosphate labeling, cells extracts were immunoprecipitated with the anti-RXRα monoclonal antibody mAb4RX3A2 and resolved by SDS-PAGE followed by autoradiography and Western blotting with RPRXα(A).

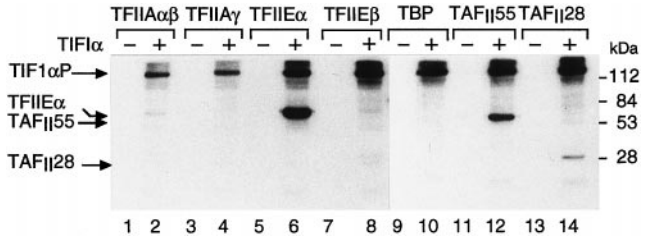

FIG. 4. **TIF1α phosphorylates certain transcription factors *in vitro*.** Purified *E. coli*-expressed recombinant transcription factors TFIIAαβ, TFIIAγ, TFIIEα, TFIIEβ, TBP, $TAF_{II}55$, and $TAF_{II}28$ (0.5 μg) were incubated in a kinase reaction in the absence (*lanes 1*, *3*, *5*, *7*, *9*, *11,* and *13*) or presence (*lanes 2*, *4*, *6*, *8*, *10*, *12,* and *14*) of immunopurified recombinant His-TIF1α. Among the factors tested, only TFIIEα (*lane 6*) and the TBP-associated factors $TAF_{II}55$ (*lane 12*) and $TAF_{II}28$ (*lane 14*) were phosphorylated by His-TIF1α.

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. 2*B*). 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. 2*C*), 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 G*X*G*XX*G, 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 [^{32}P]orthophosphate and, after disruption, were immunoprecipitated with ER or RXR antibodies. The phosphorylation of ERα was induced by estradiol (Fig. 3*A*, 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 9*C*-RA (Fig. 3*B*).

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 TFIIAαβ, TFIIAγ, TFIIEα, TFIIEβ, TBP, $TAF_{II}135$, $TAF_{II}20$, $TAF_{II}18$, $TAF_{II}28$, and $TAF_{II}55$ (31, 32) was investigated *in vitro* (Fig. 4*A*) (data not shown). Among these factors, only TFIIEα and the TBP-associated factors $TAF_{II}28$ and $TAF_{II}55$ were phosphorylated by immunopurified recombinant His-TIF1α (Fig. 4*A*, *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 autophosphorylates and phosphorylates transcription factors such as TFIIEα, $TAF_{II}28$, and $TAF_{II}55$ *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 250-kDa TATA-binding protein-associated factor of RNA polymerase II, $TAF_{II}250$, which contains a bromodomain, has been shown to possess a histone acetyltransferase activity and two kinase domains (38), whereas the Brahma proteins (BRM or SNF2α and BRG1 or SNF2β) were found to be phosphoproteins (39). Thus, TIF1α can be added to the growing number of bromodomain-containing proteins that possess a kinase activity and are themselves phosphorylated.

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.

Acknowledgments—We thank Y. Lutz for production of antibodies, I. Kolb-Cheynel for baculovirus production, and P. Oberling for peptide synthesis. We are grateful to I. Davidson for critical reading of the manuscript. We also thank the cell culture group for maintaining and providing cells and the illustration and secretarial staff for help in preparing the manuscript.

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The Putative Cofactor TIF1α Is a Protein Kinase That Is Hyperphosphorylated upon Interaction with Liganded Nuclear Receptors

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J. Biol. Chem. 1998, 273:16199-16204.
doi: 10.1074/jbc.273.26.16199