TIP30 Interacts with an Estrogen Receptor α-interacting Coactivator CIA and Regulates c-myc Transcription*

Received for publication, February 18, 2004, and in revised form, April 5, 2004
Published, JBC Papers in Press, April 8, 2004, DOI 10.1074/jbc.M401809200

Chao Jiang‡‡, Mitsubiro Ito‡‡, Valerie Piening§§, Kristy Bruck‡‡, Robert G. Roeder**, and Hua Xiao††††

From the ‡‡Eppley Institute for Cancer Research and the §§Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, Nebraska 68198-7696, the ‡Division of Hematology/Oncology, Department of Medicine, Kobe University School of Medicine, 751 Kusunoki-cho, Chuo-ku Kobe 650-0017, Japan, and the **Laboratory of Biochemistry and Molecular Biology, the Rockefeller University, New York, New York 10021

Deregulation of c-myc expression is implicated in the pathogenesis of many neoplasias. Estrogen receptor α (ERα) can increase the rate of c-myc transcription through the recruitment of a variety of cofactors to the promoter, yet the precise roles of these cofactors in transcription and tumorigenesis are largely unknown. We show here that a putative tumor suppressor TIP30, also called CC3 or Htatip2, interacts with an ERα-interacting coactivator CIA. Using chromatin immunoprecipitation assays, we demonstrate that TIP30 and CIA are distinct coactivators that are dynamically associated with the promoter and downstream regions of the c-myc gene in response to estrogen. Both TIP30 and CIA are recruited to the c-myc gene promoter by liganded ERα in the second transcription cycle. TIP30 overexpression represses ERα-mediated c-myc transcription, whereas TIP30 deficiency enhances c-myc transcription in both the absence and presence of estrogen. Ectopic CIA cooperates with TIP30 to repress ERα-mediated c-myc transcription. Moreover, virgin TIP30 knockout mice exhibit increased c-myc expression in mammary glands. Together, these results reveal an important role for TIP30 in the regulation of ERα-mediated c-myc transcription and suggest a mechanism for tumorigenesis promoted by TIP30 deficiency.

Estrogen plays an important role in the development and maintenance of the mammary glands, as well as various other tissues, and in numerous human diseases that include breast and endometrial cancer, cardiovascular disease, and osteoporosis (1–5). Most of the effects of estrogen are facilitated by estrogen receptor α (ERα),† which controls the expression of a number of hormone-responsive genes (5, 6), including the c-myc gene, which is important for cell proliferation (7–10). ERα, like many other nuclear receptors, contains two intrinsic transcriptional activation domains, designated AF-1 and AF-2 (3, 11, 12). The function of AF-1 is estrogen-independent, whereas the activity of AF-2 is estrogen-dependent. AF-1 and AF-2 activities show promoter context and cell type specificity and can act synergistically to activate transcription (5).

Although the precise mechanisms by which ERα regulates gene expression are still not clearly defined, there is solid evidence suggesting that ERα executes its effects by directing cyclical and combinatorial recruitment of cofactors on promoters (13–17). Most ERα-interacting coactivators identified thus far are also able to interact with many other members of the nuclear receptor superfamily and have generalized functions, hence affecting transcriptional activation mediated by a wide spectrum of nuclear receptors (17–21). More recently, Giguere and co-workers (22) identified a novel nuclear receptor coactivator, called CIA (coactivator independent of AF-2 function). CIA was shown to interact with ERα and ERβ in a ligand-dependent manner but not with other members of the nuclear receptor family. Consistent with its binding specificity, CIA was found to potentiate transcriptional activation by the ER but not by other nuclear receptors (22). Nevertheless, the precise mechanism by which CIA enhances transcription remains unknown. CIA may represent a novel class of ligand-dependent ER coactivators that are independent of AF-2 function.

We previously purified a protein, TIP30 (Tat-interacting protein 30) that interacts specifically with the activation domain of Tat (23). TIP30 is identical to CC3, which is absent in highly metastatic human small cell lung carcinoma (24). TIP30/CC3 has been proposed to function as a metastasis suppressor via its ability to promote apoptosis and inhibit angiogenesis (24–27). Consistent with this hypothesis, ectopic expression of TIP30 was found to elevate the expression of a subset of pro-apoptotic genes (27) and angiogenic inhibitors and to down-regulate the expression of certain angiogenic stimulators (25). Moreover, deletion of one or both alleles of Tip30 results in spontaneous development of hepatocellular carcinomas and other tumors in mice at a relatively long latency (28). Reduced expression of Tip30 is observed in 33% of human hepatocellular carcinomas, and mutations in the Tip30 gene that caused the instability or abnormal cellular distributions of the Tip30

of cDNA ends; RT, reverse transcriptase; TR, thyroid receptor; CBP, CREB-binding protein; CRED, CAMP-response element-binding protein; TRE, thyroid response element; GAL4, yeast transcription factor GAL4 DNA-binding domain; RXR, retinoid X receptor; ER, estrogen response element.

This paper is available on line at http://www.jbc.org

27781
protein were identified in some of the human hepatocellular carcinoma specimens (28). These data further suggest that Tip30 is a tumor suppressor.

In this study, we describe the cloning and characterization of a Tip30-interacting protein identical to CIA and provide evidence that Tip30 is an important regulator of ERα-mediated c-myc transcription. This study defines a new pathway for regulating expression of the c-myc gene and possibly other ERα target genes that are involved in tumorigenesis.

EXPERIMENTAL PROCEDURES

Protein Purification and Chromatography—The HeLa cell line (HeLa-FF30) stably expressing FLAG-tagged Tip30 was obtained by the introduction of pCIN4-FLAG-tagged Tip30 into HeLa S3 and selection in a G418-containing medium (27). Nuclear extracts were prepared as described previously (29) with the following modifications. During purification procedures, HEPES-HCl, pH 7.9, was used in buffers instead of Tris-HCl. Nuclear pellets were resuspended in a low salt buffer containing sulfoconcinimidylpropionate (Pierce), transferred to a homogenizer, and mixed with six strokes of a loose pestle. The suspensions were then transferred to glass beakers and dispersed with a stirring bar. The high salt buffer was slowly added into the mixtures. The cross-linking reactions were incubated at 4°C for 2 h and then stopped by adding 8 Triton-X, pH 8.0, to a final concentration of 20 mM. The extracts were centrifuged at 15000 rpm for 30 min and stored at −80°C. For cotransfection, 100 ml of nuclear extract prepared from the HeLa-FF30 cells was mixed with 200 ml of anti-FLAG epoite immunoaffinity matrix M2 (Sigma) in BC450 containing 0.1% Nonidet P-40 and 0.45M KCl. The matrix was washed extensively with BC450 containing 0.1% Nonidet P-40 and 0.5M KCl and eluted with 1 ml of BC1000 containing 200 µg/ml FLAG peptide and 1M KCl. The eluted proteins were concentrated and resolved on SDS-PAGE and stained with silver or Coomassie Blue. The indicated bands were excised from the gel and digested with endoproteinase C. The resulting peptides were isolated by high pressure liquid chromatography and subjected to mass spectrometry analysis (Core Facility, Rockefeller University). Affinity chromatography was carried out as described previously (23).

Cloning of the CIA Gene—An expressed sequence tag clone (DKFZp434J208) was initially identified by data base search using two peptides (NPQADAMVLLV and DLDRFD) from amino acid sequence and peptide mass data from mass spectrophotometer analysis of a Tip30-interacting protein (65 KDa). The expressed sequence tag clones (IMAGE: 1185534 and 1335209), containing similar cDNA sequences, were identified and purchased from ATCC and sequenced. Primers were designed for 5'-RACE and 3'-RACE PCRs according to the sequence of the insert cDNA. The HeLa cDNA library (Clontech) was used as a template for 5'-RACE and 3'-RACE. The experiments were carried out according to the manufacturer’s instructions (Invivogen). The resulting DNA fragments were subcloned into a pCR2.1 vector by PCR kits (Invitrogen). Several cDNA fragments were amplified and sequenced by the core facility at the University of Nebraska Medical Center, and the resulting sequences were used for searching the expressed sequence tag clones and known genes in GenBank (32). The 856-bp cDNA containing the translational start codon was amplified using PCR primers (primary primer, 5' -ATCTCTACTATGTGTGTG-3' and secondary primer, 5' -GGTAAGATCCCAT-3'). The resulting DNA fragment was sequenced by the core facility at the University of Nebraska Medical Center.

Plasmids and Antibodies—the pDNA1-3.CIA plasmid was generated by inserting the cIA cDNA, excised from pcR2.1-CIA with BglIII, into pcDNA1 at the BamHI site. Antibodies against human cIA and possible other ERa target genes were used for transfection efficiency, and activities of firefly and Renilla luciferase were divided by the total number of epithelial cells counted.

Histology and Immunohistochemistry—Mammary gland 4 was removed and fixed with 10% buffered-formalin and embedded in paraffin blocks. The sections were deparaffinized, rehydrated, and stained with hematoxilin and eosin. For immunohistochemistry staining, unstained sections were dehydrated and incubated overnight at 4 °C with anti-CIA antibody (Upstate Biotechnology, Inc.), and staining was developed by using vectastain Elite ABC kit (Vector Laboratories) according to the manufacturer’s instructions and then counter-stained with hematoxilin and eosin. The number of Myc nuclear positive epithelial cell was divided by the total number of epithelial cells counted.

RNA Isolation and RT-PCR—The abundance of c-myc mRNA in the tissue was measured by a semiquantitative RT-PCR analysis using β-actin mRNA as a control. Total RNA was isolated from mammary glands of 8-week-old virgin mice using Trizol reagent. 2 µg of DNA-free total RNA was reverse transcribed into cDNA using oligo(dT)15 and SuperScript II RT (Invitrogen) following the manufacturer’s instructions. The same amounts of resulting cDNA were used for PCR amplification. The primers used for PCR and their sequences are as follows: c-myc; sense, 5'-CGACTGACGACCACTATCTGATTCGAGGAGC-3', antisense, 5'-TGCACGGAGGGTGCTGCAAG-3'; β-actin, sense, 5'-CCACACAGAAGTCTGGCCGCACACCAGAC-3', antisense, 5'-TGGAGGACGAGGGTCTGGAGGAGGAGG-3'; and cyclin B1, sense, 5'-ACCTACAGGCGTGAAGTACTGAGT-3', antisense, 5'-CACCAGGACCACCGTGTCTGTGC-3'. The PCR product was analyzed in 1.2% agarose gel following ethidium bromide stain.
RESULTS

Identification of a TIP30-interacting Protein and Cloning Its Full-length Cognate cDNA—The discovery of roles of TIP30 in the control of expression of genes involved in apoptosis and angiogenesis indicates that TIP30 may interact with transcription factors to regulate gene expression. To identify and affinity purify cellular proteins that interact with TIP30, we first established HeLa cell lines that stably expressed FLAG-tagged wild type TIP30. After preparation of nuclei from these cells, nuclear proteins were cross-linked by mixing the nuclei with the water-soluble cross-linker sulfosuccinimidylpropionate. Sulfosuccinimidylpropionate-treated nuclear extracts were made and subjected to affinity purification with anti-FLAG M2 beads. The bound proteins were eluted with FLAG peptide and analyzed by SDS-polyacrylamide gel electrophoresis after cleaving the cross-linker. As shown in Fig. 1A, three proteins were communopurified with TIP30 and designated TIP30-interacting proteins. These three proteins were not immunoprecipitated with control anti-hemagglutinin beads. Amino acid sequencing of one of these interacting proteins (65 kDa), designated TIP30-interacting protein 1 (TIPIF-1), yielded two peptide sequences (NMPQADAMLVAR and DLRDFR). A HeLa cell-derived 2.2-kb cDNA encoding TIPIF-1 was cloned and sequenced (GenBank™ accession number AF470686). Sequence analysis revealed that it encodes a 587-amino acid protein with a tract of Arg-Asp residues in the amino-terminal region. The repeat Arg-Asp residues were previously identified in the splicing factor U1 70K and the RD protein, a component of the negative elongation factor NELF (32). TIPIF-1 also contains a receptor-binding motif known as the LXXLL motif (33, 34) and seven repeats of a short sequence motif (RDLRDF/H/FIR) that is present in subunit 10 of mouse translation initiation factor 3. Following cloning of the TIPIF-1 cDNA, a database search revealed identity with the estrogen receptor coactivator CIA (22). The reported CIA cDNA, which was isolated from a human fetal kidney cDNA library, contains a 620-residue open reading frame lacking a translational stop codon at the 5′ end. In contrast, the open reading frame in our TIFIF-1 cDNA starts at a methionine residue located immedi-

ately after two in-frame stop codons, indicating that it encodes a full-length CIA. The sequence discrepancy in the 5′-untranslated region between TIPIF-1 and CIA may be due to alternative splicing products in HeLa and kidney cells, because the cDNA encoding CIA was cloned from the human fetal kidney cDNA library (22). We now refer to TIPIF-1 as CIA. To ascertain whether CIA interacts with TIP30, we tested the binding of TIP30 from nuclear extracts to a GST-CIA fusion protein. As shown in Fig. 1B, TIP30 was detected in the eluate from the GST-CIA affinity column but not in the eluate from the control GST column. In addition, we also tested the binding of in vitro translated CIA to GST-TIP30 using a pull-down assay. As shown in Fig. 1C, CIA bound GST-TIP30 but not GST control protein. By contrast, BAX protein, a pro-apoptotic protein used as a negative control was not bound by GST-TIP30. Collectively, these results indicate that CIA interacts with TIP30.

Estrogen Regulates TIP30 and CIA Occupancy of the c-myc Gene in Breast Cancer MCF-7 Cells—Transcription of the c-myc gene is controlled by transcription factors that interact with numerous positive and negative regulatory elements in the c-myc promoter regions (35). ERα can stimulate c-myc transcription by interacting with an estrogen-responsive element of the c-myc promoter (8). Using ChIP assays, a previous study has established that a number of ERα-interacting coactivators are recruited to the promoters of endogenous estrogen-responsive target genes, including c-myc, following estrogen treatment (15). If CIA and TIP30 are specific cofactors for ERα, they might also be associated with ERα on endogenous estrogen-responsive target genes. To test this possibility, we used the same ChIP assay (15) to determine whether CIA and/or TIP30 is recruited to the promoter of the c-myc gene in the estrogen-dependent human breast cancer cell line MCF-7. Fig. 2B shows that, as previously reported, the anti-ERα antibody precipitated a c-myc promoter fragment containing P1 and P2 promoters in the presence (lane 6) but not in the absence (lane 5) of E2. In contrast, the c-myc promoter was precipitated by anti-TIP30 antibody (lane 3) but much less so by anti-CIA antibody (lane 1) in the absence of E2. E2 increased the asso-

FIG. 1. Purification of TIP30-interacting proteins. A, TIP30-interacting proteins were purified from HeLa nuclear extracts as described under “Experimental Procedures.” The final preparations were resolved in 10% SDS-PAGE and stained with silver (lane 2). These three proteins were not communoprecipitated if anti-hemagglutinin epitope immunoprobness matrix was used (lane 1). B, CIA binds to TIP30 from HeLa nuclear extracts. 300 μl of HeLa nuclear extracts was chromatographed on 30 μl of GST and GST-CIA columns. The columns were washed with BC100 extensively and eluted with 120 μl of BC1000. The proteins eluted from the columns were separated with SDS-PAGE and immunoblotted with anti-TIP30 antibodies. C, TIP30 interacts with in vitro synthesized CIA. Pull-down assays were performed as described under “Experimental Procedures.” The bound proteins were analyzed by 12.5% SDS-PAGE and autoradiography. The input represents 17% of the [35S]methionine-labeled CIA used in the assay (lane 1). CIA, but not a pro-apoptotic protein BAX, interacts with GST-TIP30 (lane 3) but not with GST (lane 2).
The Roles of TIP30 and CIA in c-myc Transcription

Fig. 2. Recruitment of CIA and TIP30 to the endogenous c-myc gene. A, schematic representation of the c-myc gene and primers for gene-specific PCR. Transcription initiation site and start codon are indicated. The solid bars and numbers indicate the positions of the primers corresponding to the regions of the c-myc gene. The end of c-myc mRNA is at position 5190 or 5350 (7). B, assembly of CIA and TIP30 on the c-myc gene in the first transcription cycle upon E2 induction. MCF-7 cells were treated with $10^{-8}$ M E2 for 45 min. Top panel, recruitment of ERα and CIA to the promoter of the c-myc gene. The 307-bp amplified DNA fragment corresponds to the region of the promoter. Middle panel, association of CIA with exon 2 that is 2 kb downstream of the initiation site; bottom panel, or exon 3 that is 4 kb downstream of the initiation site. The 452- or 355-bp amplified DNA fragments correspond to the region of exon 2 or exon 3, respectively. C, ERα, TIP30, and CIA are not assembled on Cyc B1 promoter. Primer pairs covering −75 to +185 region (33) were used for ChIP analysis. Pol II, polymerase II. D, ChIP analysis with a normal rabbit preimmune serum (NRS). E, CIA and TIP30 protein levels in MCF-7 cells in the absence of E2 (lanes 9 and 10). However, ERα, TIP30, and CIA were not associated with the promoter in either the presence or absence of E2 (Lanes 3–6). In addition, nonspecific antibodies from a preimmune rabbit serum did not precipitate the DNA elements of the c-myc gene (Fig. 2D). The protein levels of TIP30 and CIA in MCF-7 cells are significantly changed after E2 treatment (Fig. 2E), suggesting that E2-regulated association of TIP30 and CIA with the c-myc gene is not due to the influence of TIP30 and CIA expression by E2. Therefore, associations of these proteins with the c-myc gene are specific.

To determine whether the occupancy of the c-myc gene by TIP30 and CIA requires elongating RNA polymerase II in estrogen-dependent and -independent transcription, MCF-7 cells were treated with α-amanitin, which specifically inhibits RNA polymerase II elongation (15, 36), and then subjected to ChIP analysis. Consistent with the preceding observations, the occupancy of the c-myc promoter by TIP30 and CIA was not affected by α-amanitin (Fig. 2F). In contrast, the occupancy of...
Fig. 3. Occupancy of the c-myc gene by TIP30 and CIA in the second cycle transcription as measured by ChIP. MCF-7 cells were treated with 10^-8 M E2 and harvested at different time points. Antibodies used for immunoprecipitation are indicated. A, assembly of TIP30 and CIA on the promoter in the second transcription cycle. The 307-bp amplified DNA fragment corresponds to the region of the promoter. As was shown previously, ERα and CBP. Prolonged estrogen treatment even further increases association of CIA and TIP30 with the coding region of the c-myc gene (Fig. 3B).

Loss of TIP30 Increases ERα-mediated Transcription—To verify that the effect of TIP30 on ERα-mediated transcription is not limited by its overexpression, we next examined whether the loss of TIP30 expression affected ERα-mediated transcription by performing transient transfection assays in Tip30-/-, Tip30+/−, and Tip30+/+ MEFs. Consistent with the preceding results, Fig. 5A shows that the loss of TIP30 results in an increased ERα-mediated transcription from the artificial ERE-binding promoter containing three EREs (left panel). Coexpression of either TIP30 alone or TIP30 and CIA in Tip30-/- MEFs results in inhibition of ERα-mediated transcription (right panel). In contrast, the loss of TIP30 had a minimal effect on

the coding region of the c-myc gene by TIP30 and CIA was observed at 150 min following E2 treatment (Fig. 3B). In contrast, much less CBP was detected to associate with exon 2 upon E2 induction (Fig. 3B). Therefore, these results suggest that estrogen increases the recruitment of CIA to but decreases dissociation of TIP30 from the c-myc promoter region during the first transcription cycle. Unlike the other known ERα coactivators, which are released during elongation, CIA shows an increased association with the coding regions following E2 treatment. However, in the second cycle of estrogen-induced transcription, both CIA and TIP30 are associated with the promoter as was observed for ERα and CBP. Prolonged estrogen treatment even further increases association of CIA and TIP30 with the coding region of the c-myc gene (Fig. 3B).

Ectopic Expression of CIA and TIP30 Inhibits ERα-mediated Transcription—To test whether CIA and TIP30 function as coregulators of ERα in the transcription of the c-myc gene, we transfected COS-1 cells with vectors expressing ERα, CIA, and CBP. Interestingly, the levels of endogenous TIP30 and CIA in COS-1 cells, whereas ectopic CIA had no significant effect on transcription. The kinase-defective mutant TIP30 (30) did not significantly affect ERα-dependent transcription. Surprisingly and paradoxically, coexpression of ectopic CIA with ectopic TIP30 did not reverse but instead further potentiated the inhibitory effect of TIP30 (4-fold) in ERα-mediated transcription.

To determine whether TIP30 and CIA also regulate transcription from other promoters, we performed transient transfection experiments using the luciferase reporter gene construct containing the artificial promoter either with estrogen receptor-binding sites (EREs) or with thyroid receptor (TR) α-binding sites (31). As expected, CIA potentiated ERα-mediated transcription from this promoter in the presence of E2 (Fig. 4B), but TIP30 inhibited the activity of ERα, whereas a kinase-defective mutant TIP30 (30) did not. Consistent with the observations for c-myc promoter, the activity of ERα on the ERE-binding promoter was inhibited by ectopic expression of both TIP30 and CIA. In contrast to ERα, the activity of TRα on the TRE-binding promoter was only slightly increased by cotransfection of TIP30 and/or CIA with TRα (Fig. 4C), suggesting that neither TIP30 nor CIA had a significant effect on TR-mediated transactivation.

Taken together, these data indicate that both CIA and TIP30 are important interacting factors in modulating the activity of ERα, and CIA can cooperate with TIP30 to repress ERα-mediated transcription when they are overexpressed. However, because Western blot analyses revealed that the levels of transiently expressed ectopic TIP30 and CIA were much higher than the levels of endogenous TIP30 and CIA in COS-1 cells (data not shown), it is possible that the effects of TIP30 and CIA on ERα-mediated transcription observed here may not represent their physiological functions.

The Roles of TIP30 and CIA in c-myc Transcription

Estrogen Dynamically Regulates Occupancy of the c-myc Gene by TIP30 and CIA—Occupancy of ERα and RNA polymerase II on the promoter was previously shown to peak at 30–45 min in the first transcription cycle and at 120–150 min in the second cycle following the addition of E2 (15). We therefore sought to determine the timing of TIP30 and CIA occupancy on the promoter. As was shown previously, ERα and CBP occupancy on the promoter peaked at 30–60 min in the first transcription cycle and at 150 min in the second cycle upon E2 treatment (Fig. 3A). The results obtained with CIA were similar to those obtained with ERα and CBP. Interestingly, TIP30 was dissociated with the promoter at 60 min and reassociated with the promoter at 120–150 min in the second cycle (Fig. 3A). Increased binding of TIP30 and CIA to exon 2 was
ligand-dependent transactivation by Gal4-RXRα (AF2 domain of retinoid X receptor) (19), implying that TIP30 may preferentially inhibit the transcriptional activity of ERα (Fig. 5C). Expression of TIP30 did not completely restore ERα activity in Tip30−/− MEFs. We reasoned that increased ERα-mediated transcription was partly due to TIP30 deficiency in Tip30−/− MEFs and partly due to other genetic changes that these cells may have undergone.

We also tested whether the complete absence of TIP30 in cells affects the transcriptional activity of ERα on the c-myc promoter. As expected, ERα showed a greater stimulation of the c-myc promoter in Tip30−/− MEFs than in Tip30+/− MEFs (16-fold; Fig. 5B, left panel) and Tip30+/− MEFs (3-fold lower) in the presence of E2. TIP30 loss also resulted in a 5-fold increase in estrogen-independent transcription from the c-myc promoter (Fig. 5B, left panel). This effect was due to the ab-

---

**Fig. 4. Effect on ERα-mediated transcription by overexpression of CIA and TIP30.** A, COS-1 cells were transfected with a Myc-luciferase reporter containing the human c-myc promoter (250 ng) and vectors for expressing ERα (25 ng), CIA (50 ng), TIP30 (50 ng), or a kinase defective TIP30 mutant (TIP30M, 50 ng) in the presence of 10−8 M E2. B, COS-1 cells were transfected with a reporter (250 ng) containing three copies of ERE-binding sites and vectors for expressing ERα (25 ng), CIA (50 ng), TIP30 (50 ng), or TIP30m (950 ng), a kinase-defective mutant (27) in the presence of 10−8 M E2 as indicated. C, COS-1 cells were transfected with a TRE-luciferase reporter (250 ng) containing five copies of TRE-binding sites and vectors for expressing human TRα (25 ng), CIA (50 ng), TIP30 (50 ng), or TIP30m (50 ng) as indicated in the presence of 10−7 M T3.
The Roles of TIP30 and CIA in c-myc Transcription

The expression of TIP30 inTip30−/− cells resulted in a 42% inhibition in transcriptional activity of ERα (Fig. 5B, right panel). The finding that deletion of the Tip30 gene elicits higher transcription from the c-myc promoter indicates that the function of TIP30 is to repress both estrogen-independent and estrogen-dependent ERα-mediated c-myc transcription.

Lack of TIP30 Increases c-myc Expression in the Mammary Gland—To demonstrate that TIP30 negatively regulates expression of the c-myc gene in vivo, we utilized a semi-quantitative RT-PCR analysis to examine the level of c-myc mRNA in the mammary gland from virgin Tip30−/− mice. As expected, a higher level of c-myc RNA was observed in mammary glands fromTip30−/− mice relative to age-matched Tip30+/+ mice (Fig. 6A). In contrast, the level of cyclin B1 mRNA (Fig. 6A), whose promoter was not bound by ERα, CIA, or TIP30 (Fig. 2C), was relatively normal in these Tip30−/− mammary glands. We next used an immunohistochemical analysis to evaluate c-myc protein expression in the Tip30−/− mammary glands. Punctate staining for c-Myc protein was detected in both Tip30+/+ and Tip30−/− mammary epithelial cells. However, 41% of the Tip30−/− mammary epithelial cells had nuclear staining for c-myc, whereas 27% of the Tip30+/+ mammary epithelial cells had nuclear staining for c-myc (Fig. 6C). We also examined ERα expression in these mammary glands using immunohistochemistry and Western blot analyses. The level of ERα and percentages of ERα nuclear staining in both Tip30+/+ and Tip30−/− mammary epithelial cells were similar (data not shown), indicating that increased c-myc expression is not due to an increased expression of ERα in the mammary epithelium. These results demonstrate that a lack of TIP30 results in increased c-myc expression in mouse mammary glands. Taking these results together, we concluded that TIP30 is a repressor of ERα-mediated transcription of the c-myc gene.

DISCUSSION

ERα-mediated transcription requires corepressors that interact with ERα and enhance its transcriptional activity. Although most ER corepressors identified thus far associate with the AF-2 domain and enhance the transcriptional activity of many nuclear receptors, as well as nonreceptor activators (4, 14, 37), the unique coactivator CIA specifically enhances ERα activity independent of AF-2 function (22). In the present study, we have demonstrated the interaction between a puta-
Figure 6. Loss of TIP30 increases c-myc expression in mammary glands. A, loss of TIP30 increases the levels of c-myc mRNA in mammary glands. RNA was isolated from mammary glands of 8-week-old virgin mice (C) and 8-week-old virgin mice (B) and Tip30−/− mice. RT-PCR analysis was used to monitor expression of the c-myc, cyclin B1, and β-actin genes. B–D, loss of TIP30 increases c-myc-stained epithelial cells in mammary glands. Representative immunostaining for c-myc on mammary gland sections from 8-week-old Tip30−/− (B) and Tip30−/− virgin mice (C) are shown. The bar graph indicates the percentage of c-myc nuclear-positive epithelial cells (D). The results represent two 8-week-old virgin mice for each genotype. About 500 epithelial cells were counted in the mammary gland section for each animal.

The Roles of TIP30 and CIA in c-myc Transcription

Enhanced ERα-mediated transcription in transient transfection assays (Fig. 3) and increased c-myc expression in mammary glands (Fig. 4). Therefore, we conclude that interaction between TIP30 and CIA is biologically relevant, and TIP30 is a repressor of ERα-mediated transcription of the c-myc gene.

Although CIA is not related to any known proteins, it contains a receptor-binding motif known as the LXXLL motif and a motif consisting of Arg-Asp repeats (22). Interestingly, the Arg-Asp repeats were previously found in a putative RNA-binding component (RD) of NELF (32) that interacts with RNA polymerase II and represses transcription. Because CIA, unlike other ER-interacting coactivators, is not released during elongation (Fig. 3), it may bind to RNA via the Arg-Asp repeats to regulate elongation. It is interesting in this regard that estrogen promotes occupancy of CIA on the promoter and the coding regions of the c-myc gene. The precedent for this type of assembly of transcription factors is observed in galactose activation of GAL1 and heat shock activation of HSP82 in yeast and hsp70 in HeLa cells. Elongation factors such as Spt5 (38) and Sug1/Rpt6 (39, 40) are associated with both promoters and open reading frames of transcribed genes in yeast. Transcription activation induces occupancy of those factors on the GAL1 promoter and the open reading frame. Therefore, we propose that CIA may be an elongation factor that is specifically involved in the transcription of ERα-targeted genes in mammary cells. TIP30 and CIA may represent a novel class of coregulators for ERα.

The c-myc gene is controlled by numerous transcription factors through upstream sequences of its P1 and P2 start sites (7, 35). Previous studies have established that ERα increases c-myc transcription through a noncanonical ERE element (8). The experiments in the current study were designed to first determine the role of TIP30 and CIA in ERα-mediated c-myc transcription. Our data clearly demonstrate that TIP30 and CIA are involved in the regulation of c-myc transcription. It should be emphasized that the inhibition of ERα-mediated transcription by TIP30 is not caused by a nonspecific repression, because TIP30 overexpression or loss of expression failed to alter transactivation by other nuclear receptors under the same conditions. Overall, previous (22) and current studies have suggested complicated roles of TIP30 and CIA in ERα-mediated transcription, although it remains unclear precisely how TIP30 and CIA function in this process. However, based on our data, we hypothesize that TIP30 and CIA may act as repressors of c-myc transcription. In this scenario, the binding of TIP30 to the c-myc promoter in the absence of E2 might lead to phosphorylation of the CTD of RNA polymerase II (27) during the formation of preinitiation complexes. This in turn might destabilize the association of RNA polymerase II with coactivators such as AIB1, p300, pCAF, CBP, and TRAPs (15) at the initiation step. In this regard, an inhibitor of RNA polymerase II elongation, α-amanitin, (15, 36), which inhibited the association of RNA polymerase II with the coding region of the c-myc gene, also abolished the association of TIP30 and CIA (Fig. 2F), suggesting that association of TIP30 and CIA with the coding region is RNA polymerase II-dependent. In addition, TIP30 was previously shown to phosphorylate the CTD in vitro (27), and the kinase-defective TIP30 mutant failed to repress ERα-mediated transcription (Fig. 4B). In the first transcription cycle, CIA may facilitate displacement of TIP30 from the promoter, possibly through a transient interaction with TIP30, and subsequently associate with elongation complexes. Alternatively, ERα and ERα-recruited factors could also facilitate TIP30 displacement from the promoter, with the observed TIP30-CIA interactions being more relevant to their mutual presence in transcription elongation complexes in the absence
of estrogen as well as in the second cycle transcription complexes following estrogen treatment. In the second transcription cycle, CIA may cooperate with Tip30 to repress transcription. In support of this possibility, CIA was previously shown to inhibit transcription of a Gal-driven promoter and observations of RNA polymerase II support this hypothesis. In summary, we have identified a TIP30-interacting protein and CIA directly regulate the transcription of the c-myc gene. Like other ERα cofactors (14, 15), TIP30 and CIA are recruited by ERα on the c-myc promoter in a c cyclic fashion. Consequent on its effect-mediated c-myc transcription, loss of Tip30 increases c-myc expression in the mammary gland of mice. We thank the Core Facility of the Rockefeller University for the amino acid sequence of CIA protein and K.-U. Wagner, Z. X. Wang, C. M. Eisen, and A. Diehl for technical help and useful discussions and critical reading of the manuscript.

REFERENCES
1. Couse, J. F., and Korach, K. S. (1999) Ann. Endocrinol. 60, 143–148
2. Couse, J. F., and Korach, K. S. (1999) Endocr. Rev. 20, 358–417
3. Mangelsdorf, D. J., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., and Chambon, P. (1995) Cell 83, 835–839
4. McKenna, N. J., Xu, J., Nawaz Z., Tsai, S. Y., Tsai, M. J., and O’Malley, B. W. (1999) J. Steroid Biochem. Mol. Biol. 69, 3–12
5. Nilsson, S., Makela, S., Treuter, E., Tujague, M., Thumen, J., Andersson, G., Enmark, E., Petterson, K., Warner, M., and Gustafsson, J. A. (2001) Physiol. Rev. 81, 1535–1565
6. Planas-Silva, M. D., Shang, Y., Donaher, J. L., Brown, M., and Weinberg, R. A. (2001) Cancer Res. 61, 3854–3862
7. Battey, J., Moulding, C., Taub, R., Murphy, W., Stewart, T., Potter, H., Lenoir, G., and Leder, P. (1983) Cell 34, 779–787
8. Dubik, D., and Shin, R. P. (1992) Oncogene 7, 1587–1594
9. Leder, A., Pattengale, P. K., Kuo, A., Stewart, T. A., and Leder, P. (1986) Cell 45, 485–495
10. Liao, D. J., and Dickson, R. B. (2000) Endocr. Relat. Cancer 7, 143–164
11. Tena, L., White, J., Broc, C., Tasset, D., Webster, N., Scheer, E., and Chambon, P. (1989) Cell 50, 477–487
12. Tsai, M. J., and O’Malley, B. W. (1994) Annu. Rev. Biochem. 63, 451–486
13. Barakow, D., Crofts, L. A., Chang, C. P., and Freedman, L. P. (2002) J. Biol. Chem. 275, 13459–13462
14. Metivier, R., Penet, G., Hubsch, M. R., Reid, G., Brand, H., Kes, M., and Giguere, V. (2003) Cell 114, 751–763
15. Shang, Y., Hu, X., Difrancesco, J., Lazar, M. A., and Brown M. (2000) Cell 103, 834–852
16. Shang, Y., and Brown, M. (2002) Science 295, 2465–2468
17. Xu, L., Glass, C. K., and Rosenfeld, M. G. (1999) Curr. Opin. Genet. Dev. 9, 140–147
18. Freedman, L. P. (1999) Trends Endocrinol. Metab. 10, 403–407
19. Ito, M., Okano, H. J., Darnell, R. B., and Roeder, R. G. (2002) EMBO J. 21, 3446–3475
20. Rang, Y. K., Guermah, M., Yuan, C. X., and Roeder, R. G. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 8642–8647
21. Freedman, L. P. (1999) Annu. Rev. Biochem. 70, 475–501
22. Sauve, F., McElroom, L. D., Gallant, J., Moraitis, A. N., Labrie, F., and Giguere, V. (2001) Mol. Cell. Biol. 21, 343–355
23. Xiao, H., Tao, Y., Greenblatt, J., and Roeder, R. G. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 2146–2151
24. Shitvelman, E. (1997) Oncogene 14, 2167–2173
25. Nica obligatory, R., and Shitvelman, E. (2001) Oncogene 20, 270–275
26. Whitman, S., Wang, X., Schalaby, R., and Shitvelman, E. (2000) Mol. Cell. Biol. 20, 583–593
27. Xiao, H., Palhan, V., Yang, Y., and Roeder, R. G. (2000) EMBO J. 19, 956–963
28. Ito, M., Jiang, C., Krumm, K., Zhang, X., Pech, J., Zhang, L., Guo, Y., Roeder R. G., and Xiao, H. (2003) Cancer Res. 63, 8763–8767
29. Digman, J. D., Lehebiva, R. M., and Roeder, R. G. (1983) Nucleic Acids Res. 11, 1475–1489
30. Xiao, H., Tao, Y., and Roeder, R. G. (1999) J. Biol. Chem. 274, 3937–3940
31. Yuan, C. X., Ito, M., Fandell, J. D., Fu, X., and Roeder, R. G. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7059–7064
32. Yamaguchi, Y., Takagi, T., Tada, T., Yano, K., Furuya, A., Sugimoto, S., Hasegawa, J., and Handa, H. (1999) Cell 97, 41–51
33. Heerey, D. M., Kalkhoven, E., Hoare, S., and Parker, M. G. (1997) Nature 387, 733–736
34. Le Deaurain, B., Nelsen, A. L., Garnier, J. M., Ichinose, H., Jeanmougin, F., Lussou, B., and Chambon, P. (1998) EMBO J. 17, 6701–6715
35. Spencer, C. A., and Groudine M. (1990) Anti. N. Y. Acad. Sci. 599, 12–28
36. Rudd, M. D., and Luse, D. S. (1996) J. Biol. Chem. 271, 21549–21558
37. McCormack, S. J., Weaver, Z., Deming, S., Natarajan, G., Torri, J., Johnson, M. D., Liyanage, M., Ried, T., and Dickson, R. B. (1998) Oncogene 16, 2755–2766
38. Pokholok, D. K., Hannett, N. M., and Young, R. A. (2002) Mol. Cell 9, 799–809
39. Ferdous, A., Gonzalez, F., Sun, L., Kodake, T., and Johnston, S. A. (2001) Mol. Cell 7, 981–991
40. Gonzalez, F., Delahodde, A., Kodake, T., and Johnston, S. A. (2002) Science 296, 548–550
41. Cardiff, R. D., Anver, M. R., Gusterson, B. A., Hennighausen, L., Jensen, R. A., Merino, M. J., Rehm, S., Russo, J., Tavassoli, F. A., Wakefield, L. M., Ward, J. M., and Green, J. E. (2000) Oncogene 19, 968–983
42. Cunha, G. R., Wiesen, J. F., Webster, N., Hennighausen, L., Jensen, R. A., Anver, M. R., Gusterson, B. A., Hennighausen, L., Jensen, R. A., Merino, M. J., Rehm, S., Russo, J., Tavassoli, F. A., Wakefield, L. M., Ward, J. M., and Green, J. E. (2000) Oncogene 19, 968–983
43. D’Cruz, M., Gunther, E. J., Boster, R. B., Hartman, J. L., Sintasath, L., Moody, S. E., Cox, J. D., Ha, S. I., Belka, G. K., Golant, A., Cardiff, R. D., and Chodosh, L. A. (2001) Nat. Med. 7, 235–239

Acknowledgments—We thank the Core Facility of the Rockefeller University for the amino acid sequence of CIA protein and

J. Pecha, C. Jiang, and H. Xiao, manuscript in preparation.
TIP30 Interacts with an Estrogen Receptor α-interacting Coactivator CIA and Regulates c- myc Transcription
Chao Jiang, Mitsuhiro Ito, Valerie Piening, Kristy Bruck, Robert G. Roeder and Hua Xiao

J. Biol. Chem. 2004, 279:27781-27789.
doi: 10.1074/jbc.M401809200 originally published online April 8, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M401809200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 43 references, 14 of which can be accessed free at
http://www.jbc.org/content/279/26/27781.full.html#ref-list-1