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Nuclear structure-associated TIF2 recruits glucocorticoid receptor and its target DNA

Hidesato Ogawa,[a,b,f,*] Ruth T. Yu,[a,c,1] Tokuko Haraguchi,[d] Yasushi Hiraoka,[d] Yoshihiro Nakatani,[e] Ken-ichirou Morohashi,[f] and Kazuhiko Umesono[a,c]

[a] *Department of Biochemistry, Institute for Virus Research, Kyoto University, Kyoto, Japan*
[b] *Graduate School of Biological Science, Nara Institute of Science and Technology, Ikoma, Japan*
[c] *Graduate School for Biostudies, Kyoto University, Kyoto, Japan*
[d] *Cell Biology Group and CREST Research Project, Kansai Advanced Research Center, National Institute of Information and Communications Technology, Kobe, Japan*
[e] *Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA, USA*
[f] *National Institute for Basic Biology, Okazaki, Japan*

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Abstract

Assembly of multi-protein complexes on promoter and enhancer elements is a prerequisite for onset of gene transcription. At the beginning of this process, transcription factors are thought to act as nucleating centers for complex formation through the binding of their target DNA sequences, and thereafter recruit coactivators. Here, we investigated this process of assembly by determining the distribution of the glucocorticoid receptor (GR) and its coactivator, TIF2. Both endogenously and ectopically expressed TIF2 were shown to form foci in the nucleus, and GR could be recruited to the TIF2 foci upon GR agonist but not antagonist treatment. Moreover, we show that the coactivators, p300 and PCAF, are also recruited to the TIF2 foci. The TIF2 foci could recruit GR carrying a microinjected GR responsive element. We propose that TIF2 provides a nuclear compartment that allows the assembly of multi-protein complexes required for GR-mediated gene activation.

Keywords: TIF-2; GRIP1; Transcription complex; Nuclear matrix; Nuclear hormone receptor; Steroid receptor; Nuclear compartment; Coactivator; Cofactor

Regulation of gene transcription is a multi-step process that requires assembly of protein complexes on target promoters in response to intracellular signaling events. DNA sequence-specific transcription factors bound to the target sites have been demonstrated to nucleate the assembly of multi-protein complexes and recruit cofactors. It has been proposed that a nuclear structure provides a scaffold for this complex to execute its biological function. However, the mechanisms underlying the assembly of this multi-protein complex and its anchoring to the nuclear structure remain unclear.

Nuclear hormone receptors are a family of sequence-specific transcription factors involved in diverse aspects of growth, development, and homeostasis of higher eukaryotic organisms. They activate the transcription of discrete sets of genes, typically in response to a cognate ligand [1–4]. When bound to specific enhancer elements on DNA in an activated state, they recruit coactivators and a transcription initiation complex including RNA polymerase II to the promoter region through alterations in the state of chromatin organization [5–7]. However, the coactivators themselves are not DNA-binding proteins but rather recruit the transcription initiation complex to the promoter regions through protein–protein interactions. Thus, they are assumed to act as "adaptors" in a signaling pathway that transmits transcriptional activation signals from DNA-bound

* Corresponding author. Fax: +81-564-59-5867.
E-mail address: hidesato@nibb.ac.jp (H. Ogawa).
[1] Present address: Gene Expression Laboratory, The Salk Institute, La Jolla, CA, USA.

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activator proteins to the chromatin and transcriptional complex.

Coactivator complexes are composed of several distinct sets of proteins including SRC-1 family proteins, p300/CBP [8,9], and the p300/CBP-associated factor, PCAF [10]. The SRC-1 family comprises three structurally related but genetically distinct 160-kDa proteins: SRC-1 (also called p160 and NcoA-1), TIF2 (also called SRC-2, GRIP1, and NcoA-2), and ACTR (also called SRC-3, AIB1, RAC3, TRAM1, and pCIP) [4,5,11]. Transient expression studies have demonstrated that nuclear receptor transactivation is efficiently enhanced by any one of these coactivators. Since coactivators such as p300/CBP, PCAF, and possibly SRC-1 and ACTR possess intrinsic histone acetyltransferase activities (HAT), recruitment of the coactivators by sequence-specific transcription factors can allow them to modify chromatin organization on promoter regions. In addition, the HAT activities regulate the protein–protein interaction within transcription complexes [12–15].

Extensive cytological studies have recently revealed that nuclear hormone receptors undergo continuous exchange between specific target DNA sequences and the nuclear compartment [16–19]. In addition to these findings, ligand- or modification-induced subnuclear compartmentalization on the nuclear structure is observed as foci for certain nuclear hormone receptors, and are believed to contain the transcriptionally active forms of the receptors. However, it has not yet been demonstrated directly that such subnuclear compartments represent active transcription sites [16,20]. Therefore, the true function of these nuclear compartments remains unclear.

In this study, we found that TIF2 formed foci on the nuclear structure that efficiently recruited p300 and PCAF, forming ternary complexes, prior to hormone treatment. Furthermore, the TIF2 foci could recruit ligand activated glucocorticoid receptors (GRs) together with their target sites. The results indicated that GRs preserve their DNA-binding activity in these nuclear foci. Based on these findings, we propose a novel model for transcriptional activation by GR in which TIF2 on the nuclear structure plays a key role in recruiting activators and the target DNA.

Materials and methods

Construction of GFP fusion plasmids. An expression plasmid for a green fluorescent protein (GFP) variant, pCMX-AFAP was constructed by site-directed mutagenesis of three amino acid substitutions (S65A/Y145F/M163A) into the humanized GFP (BD Biosciences Clontech, Palo Alto, CA). This modified GFP overcomes temperature-sensitive limitations of the original GFP [21], and consequently produces an improved fluorescence intensity when expressed in cultured mammalian cells. The plasmid pCMX-GR-GFP encoding human GR fused with GFP at its C-terminus was constructed by polymerase chain reaction (PCR) amplification using the following GR-specific primers to facilitate in-frame ligation: 5′-ACCCTACTGCAGTACTCCTGGATG-3′ and 5′-CCGCCGCCTAGGACGGTGCACGGTGAAAACTACTTTGTCTTCAAA-3′. The modified GR cDNA encodes a *Pml*I site after the last coding codon (777K). A Flag-tagged TIF2 plasmid, pSG5-TIF2-fg, was generated by modification of the 3′ end of TIF2 coding sequence with the PCR primers: 5′-CCAAACCAGCTGCCTGGAATGGATATGATTAAGCAGGAGGGAGACACAACACGGAAATATTGC-3′, and 5′-GGCGGCGGATCCTTGTCATCGTCGTCCTTGTAGTCCATGCAATATTTCCGTGTTGTGTCTCC-3′. The Flag peptide sequence was localized in-frame after the TIF2 *Bst*XI site.

Cell culture. Cell cultures were prepared as described previously [22]. Briefly, HeLa and COS-1 cells were grown in 100-mm dishes with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37 °C in the presence of 5% CO_2 and passaged every 2–3 days by 1:5 dilution. For experiments with hormone stimulation, cells were cultured in media (1:4 mixture of DMEM containing 10% FBS and Opti-MEM [Gibco-BRL, Grand Island, NY]) for 2 days to reduce endogenous steroid hormones, and then cultured in 35-mm dishes with 2 ml DMEM containing 10% charcoal-treated FBS for another 2 days to deplete endogenous steroid hormones. Thereafter, the cells were treated with agonist ligand (100 nM dexamethasone (Dex) for GR or 100 nM estradiol for ER) for 60–120 min at 37 °C.

Indirect immunofluorescence labeling. Cells were grown on cover glasses in 3.5-cm dishes, fixed in PBS–4% paraformaldehyde (PFA) for 10 min at room temperature, and subsequently incubated in PBS containing 2% skim milk for another 30 min. Cells were incubated with primary antibodies in PBS–0.1% Triton X-100 (PBST) at room temperature for 1 h at 1:300 dilution. TIF2 was detected using a monoclonal anti-TIF2 antibody (clone 29; BD Transduction Laboratories). Cells were then incubated with secondary antibodies in PBS–2% BSA at 37 °C for 60 min at 1:500 dilution. We used combinations of anti-rabbit or anti-mouse goat IgGs conjugated to Alexa Fluor-488 (Molecular Probes, Eugene, OR) and anti-mouse CY3 or anti-rat CY3 IgGs (Jackson Immunoresearch Laboratories, West Grove, PA). DNA was stained with DNA-specific fluorescence dye, 4′,6′-diamidine-2-phenylindole dihydrochloride (DAPI) (Sigma Chemical, St. Louis, MO).

For microscopic observation of transfected cells, the TIF2-Flag plasmid (2.5 μg) was cotransfected into cells with/without the plasmid (0.5 μg) encoding GR-GFP or estrogen receptor (ER)-GFP using a lipofection reagent, DMRIE-C (Gibco-BRL), according to the protocol provided by the manufacturer. After 36–48 h incubation, the cells were placed in fresh Opti-MEM and cultured for 1–2 days.

The cells expressing TIF2-Flag alone or both TIF2-Flag and GFP fusions were fixed with 4% PFA in PBST for 20 min at room temperature and washed three times with PBS. After fixation, the cells were incubated with blocking buffer (1% BSA dissolved in PBS) for 1 h to prevent non-specific binding of the antibody. A primary antibody was added to cells and incubated for 20 h at 4 °C; the primary antibodies used were polyclonal antibodies specific for p300 (C-20) (Santa Cruz Biotechnology, Santa Cruz, CA, at 1:100 dilution), PCAF (C-16) (Santa Cruz; 1:50), and SMRT (N-20) (Santa Cruz; 1:100), and monoclonal antibodies specific for FLAG M2 (Sigma; at 1:500 dilution) and SV40T (Pab101) (Santa Cruz; 1:1000). The cells were then washed three times with PBST and stained with a fluorescent secondary antibody, fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (Dako), Cy3-conjugated anti-rabbit IgG or Texas Red-conjugated anti-mouse IgG (Amersham Life Science, Buckinghamshire, UK) at 1:100 dilution for 3-4 h at room temperature. Finally, the cells were washed three times with PBST, incubated with 50 ng/ml DAPI for 10 min, and mounted in the mounting reagent; PermaFluor (thermoelectron) for fluorescence microscopy.

Fluorescence microscopy. Two fluorescence microscope systems were used in this study. Zeiss Axiophoto II (Carl Zeiss, Germany) with

appropriate filters was used unless specified. For imaging using this microscope, 40× or 63× water-immersion objective lenses were used as described previously [21,22]. A Delta Vision (Applied Precision, Seattle, WA) microscope system was also used. Details of the microscope system were described previously [23,24].

Localization assay of synthetic glucocorticoid responsive element. Biotinylated oligonucleotide probes (GRE-A, 5′-bio-TTGGAACCC GGGAGAACATCATGTTCTGAATTC-3′ and GRE-B, 5′-bio-GAA TTCAGAACATGATGTTCTCCCGGGTTCCAA-3′) were synthesized. The probes were denatured at 96 °C, kept at 65 °C for 20 min, and left at room temperature for 2 h. Then, the glucocorticoid responsive element (GRE) probes (1 μg/ml in PBS) were microinjected into the nuclei of living cells expressing TIF2-Flag and GR-GFP or ER-GFP. Microinjection was carried out using a microinjection system with a micromanipulator 5171 and microinjector 5242 (Eppendorf, Hamburg, Germany) with injection pressure ($P2$) = 300 hPa and maintenance pressure ($P3$) = 50 hPa for 0.1 s. After microinjection, the cells were cultured with agonist ligand (100 nM Dex for GR or 100 nM estradiol for ER) for 60 min at 37 °C, and fixed for 20 min on ice with 4% PFA/PBS containing 0.5% Triton X-100.

Results and discussion

Ligand-dependent GR foci and ligand-independent TIF2 foci formation

Recruitment of cofactors by sequence-specific activators is a key step for transcriptional activation, given their essential role in serving as modifiers of chromatin structure and/or bridging factors between activators and the RNA polymerase II transcriptional machinery [25]. Many studies have suggested that transcription factors act in concert with specific sets of coactivators in this process. To elucidate the molecular process of complex formation between nuclear receptors and coactivators, we examined the subnuclear localization of TIF2 and GR. As shown in Fig. 1, immunofluorescence studies revealed that TIF2 was expressed endogenously in HeLa

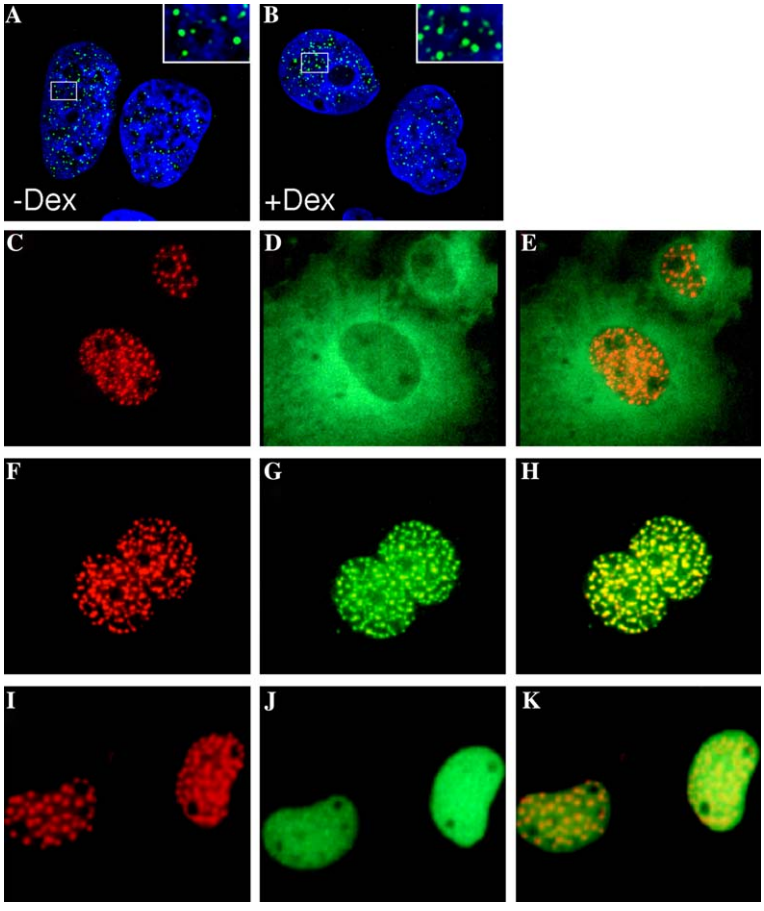

Fig. 1. Endogenous and exogenous TIF2 foci recruit agonist-bound GR. HeLa cells were stained for endogenous TIF2 with the anti-TIF2. TIF2 was visualized by the Alexa Fluor-488-conjugated secondary antibody. Nuclei were visualized by staining with DAPI. HeLa cells were cultured in the absence (A) or presence (B) of 100 nM Dex for 120 min. The insets at the upper right corner are magnifications corresponding to the white boxed area. With or without Dex treatment, the endogenous TIF2 is present in punctate nuclear structures. COS-1 cells transfected with plasmids encoding Flag-tagged TIF2 (C, F, and I) and GR-GFP (D, G, and J) were cultured in the absence (C–E) or presence of agonist ligand (F–H) for 120 min (10 nM Dex) while cultured in the presence of antagonist ligand (10 nM RU486) (I–K). The anti-Flag (M2) antibody was used in conjunction with the Texas Red-conjugated secondary antibody to visualize TIF2 proteins. GR was detected by GFP fluorescence. The merged images show colocalization of TIF2 and agonist-bound GR in the nuclei (E, H, and K). Three-dimensional optical section images were obtained on a computer-controlled, fluorescence microscope system [23,24], and digitally processed to improve resolution using an iterative deconvolution method [52].

cells and its distribution showed punctate nuclear foci (Fig. 1A). This subnuclear distribution did not change even after the cells were treated with a GR agonistic ligand, dexamethasone (Dex) (Fig. 1B). A similar distribution was observed in COS-1 cells (data not shown). These results are similar to those of Lazaro et al. [26] who described the detection of TIF2 nuclear foci in differentiating skeletal muscle cells.

To investigate the effects of the copresence of nuclear receptors on the TIF2 distribution, both GR and TIF2 were expressed in COS-1 cells. The expressed TIF2 was present in nuclear foci similar to endogenous TIF2 (Fig. 1C). Consistent with previous observations [21,22], the intracellular distribution of GR was noted in both the cytoplasm and nucleus in the absence of ligand (Fig. 1D). The nuclear distribution of GR did not overlap with that of TIF2 (Fig. 1E). After the ligand treatment, GR formed foci in the nucleus that overlapped with those of TIF2 (Figs. 1F–H). Since GR alone did not display a distinct punctate distribution upon ligand activation [21], coexpression of TIF2 seems to recruit GR to the foci. Nuclear translocation of GR was also observed after treatment with an antagonistic ligand, RU486, although the foci were never observed (Fig. 1J). Thus, a large population of GR failed to be recruited to the TIF2 foci upon the antagonist treatment (Figs. 1I–K). Likewise, agonist-dependent localization of hormone receptors to the TIF2 foci was observed for TRβ, RARα, β, AR, and ERα using GFP fusions, although these GFP fused hormone receptors appeared to localize in the nucleus even in the absence of ligand (data not shown) [27–29]. Considered together, our results suggest that ligand-binding induces translocation of certain types of nuclear receptors to the TIF2 compartment, and that antagonist-binding inhibits and/or disrupts interaction of GR with its coactivator. These findings are consistent with those of previous structural studies based on X-ray crystallography, which revealed that an interaction site for TIF2 in ERα is concealed from the antagonist-bound form [30,31].

Selective recruitment of coactivators into TIF2 foci

Since TIF2 interacts directly with p300/CBP in vitro [32], we examined whether p300 is recruited to the TIF2 foci. Endogenous p300 was barely detectable as a punctate pattern in the nucleus (Fig. 2A). In the presence of the coexpressed TIF2, however, p300 became compartmentalized to the TIF2 foci (Figs. 2B–D). Since PCAF was identified as an associate factor with p300/CBP, we examined the distribution of PCAF. Similar to p300, endogenous PCAF showed homogeneous nuclear distribution (Fig. 2E) while PCAF was observed in

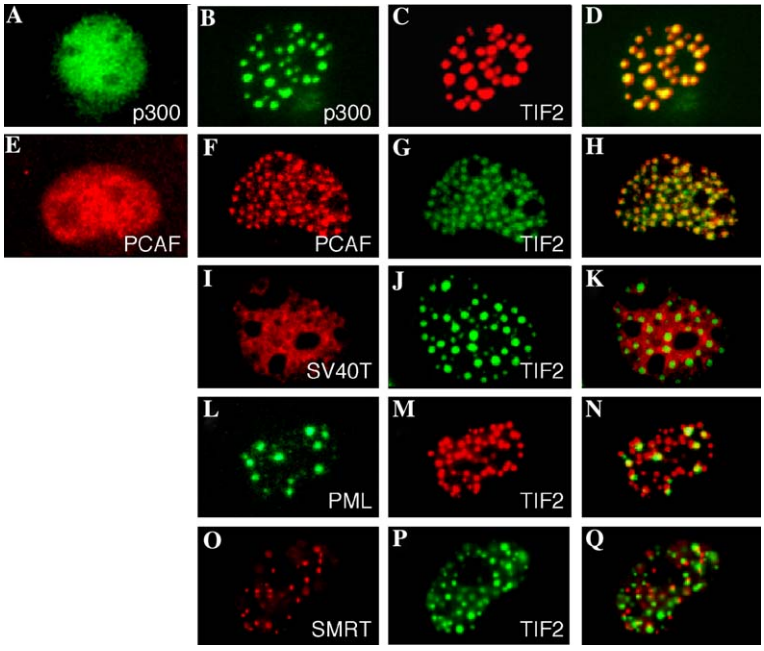

Fig. 2. Selective recruitment of coactivator into TIF2 foci in COS-1 cells. Untransfected (A,E) or TIF2-transfected (B–D, F–Q) COS-1 cells were stained for endogenous p300 with the anti-p300 (A–D) and for endogenous PCAF with the anti-PCAF (E–H). p300 was visualized by the FITC-conjugated secondary antibody and PCAF with a Cy3-conjugated secondary antibody. In untransfected cells, p300 and PCAF were detected homogeneously in the nucleus (A,E). In contrast, the merged image of the TIF2-transfected cell shows extensive overlap between both p300 and PCAF staining with the TIF2 foci (D,H). COS-1 cells were transfected with SV40T and detected with the anti-SV40T and Texas Red-conjugated secondary antibodies (I–K). Transfected PML-GFP (L–N) was visualized for GFP fluorescence and SMRT was detected with anti-SMRT and Cy3-conjugated secondary antibodies (O–Q). Although PML and SMRT formed nuclear dots, the overall patterns differed from that of TIF2 with respect to the size and number of the dots (N,Q). These optical section images were analyzed on the computer-controlled, fluorescence microscope system described in Fig. 1.

nuclear foci in the presence of the coexpressed TIF2; approximately 90% of the PCAF foci were overlapped with those of TIF2 (Figs. 2F–H). These results indicate that TIF2 recruits both p300 and PCAF to the same nuclear compartment. As in the case of GR, the overlapping localization of both p300 and PCAF with TIF2 appears reasonable if their functional interaction with TIF2 is considered.

Subsequently, we examined the distribution of PML and SV40T proteins. Although they are functionally unrelated to GR activation, both have the potential to interact with p300. Since SV40T interacts directly with p300, we examined whether SV40T is recruited to the TIF2 foci with p300 [33]. SV40T distribution was observed homogeneously in the nucleus except for the nucleoli in the presence and absence (data not shown) of the coexpressed TIF2, thus their distributions did not overlap (Figs. 2I–K). PML is known to form PML bodies in the nucleus [34,35]. Basically, this particular distribution was maintained strictly even in the presence of the coexpressed TIF2 (Figs. 2L–N). In fact, although the PML foci appear to overlap partially with the TIF2 foci, the overlapping profile was completely different from those of p300 and PCAF (Figs. 2B–D and F–H). This observation was consistent with a previous report that p300 was partially colocalized to the PML body with RNA polymerase II [36]. In addition, a corepressor for nuclear receptors, SMRT, also displayed punctate distribution, however, the SMRT foci did not overlap with those of TIF2 (Figs. 2O–Q) [37,38].

Interestingly, the distributions of p300 and PCAF were changed by the copresence of TIF2 (Figs. 2A, B and E, F). Since it was reported that p300 interacts with TIF2 [32] while PCAF interacts with p300 [39], these physical interactions might be essential for the changes in their distribution. However, it should be noted that the punctate distribution of TIF2 was not affected by the presence of the factors examined. Thus, it is reasonable to assume that TIF2 is a particular cofactor molecule that provides a scaffold for selective components of the transcriptional machinery complex.

Characterization of TIF2 foci as transcription center

To further characterize the TIF2 foci, we examined whether they disappear under stringent conditions of cellular treatments. When the TIF2-expressing cells were treated with 0.5% Triton X-100, the original punctate TIF2 foci were still retained as shown in Fig. 3A. Subsequently, the cells were treated more stringently with DNase I and washed with a high ionic buffer. Under this condition, the TIF2 foci were still retained, although chromatin DNA could no longer be detected by staining with DAPI (Figs. 3A-c and d) or Hoechst33342 (data not shown). The nuclear components retained predominantly under this stringent condition have been defined as the nuclear matrix [40]. Since it was described that some TIF2 is recovered in a soluble fraction as a complex with p300 by a high ionic treatment [41], it is unlikely that all TIF2 are retained in the foci under the stringent conditions of the present study. SMRT, which forms a nuclear punctate pattern resembling but not overlapping with that of TIF2 (Figs. 2O–Q), disappeared following treatment with 0.5% Triton X-100 (data not shown). Moreover, SRC-1, another p160 family coactivator, also formed foci with ER but could not bind the nuclear matrix [42]. These results suggest that a punctate pattern does not always indicate localization to the nuclear matrix and that the presence of TIF2 in the nuclear matrix fraction is due to the intrinsic property of the protein.

Although our initial observations indicated that the TIF2 foci colocalize with agonist-bound GR, the above study indicated that DNA is not necessarily required to maintain the TIF2 foci. It was expected that the GR-colocalizing-TIF2 foci provide an active transcription site and thus we assumed that DNA bearing a GRE might be recruited to the TIF2 foci via GR. To test this hypothesis, biotinylated oligonucleotides encoding a GRE [43] were microinjected into cells that expressed both TIF2 and GR. Upon treatment with agonist, the oligonucleotides became colocalized to the GR-TIF2 foci (Figs. 3B-a–c). This colocalization was not detected in the absence of ligand and in the presence of antagonist (data not shown). The GRE-containing oligonucleotides by themselves did not colocalize with ER on the TIF2 foci (Figs. 3B-d–f). These results indicate that GR maintains specific DNA-binding activity on the nuclear structure-associated TIF2 foci. Based on these results, TIF2 appears to form a functional nucleus of a cofactor complex and potentially recruits activated receptors with their target DNA.

Several lines of evidence support that transcriptional and post-transcriptional events take place in specific domains on nuclear structures such as the nuclear matrix, which have been termed "transcriptional factories": immunocytochemical run-on transcription assays demonstrate that nascent RNA forms a nuclear dot pattern that colocalizes with RNA polymerase II, CBP/p300, and splicing factors such as Sm antigen [36,44]. Moreover, it has been reported that RNA polymerase II and a number of transcriptional activators including AML/CBF-α, YY-1, Pit-1, and nuclear hormone receptors are associated, at least partly, with the nuclear matrix [45–47]. However, the mechanisms through which these nuclear matrix-associated factors contribute to transcription remain unclear. Our results indicate that the coactivator complex nucleated by TIF2 acts as one such "transcripcriptional factory" through association with the nuclear structure. In turn, nuclear hormone receptors and target DNA are recruited to this site in a ligand-dependent manner. In a previous report, McNally et al. [48] showed that a dynamic exchange of two distinct forms of GR, bound and unbound to its enhancer elements in chro-

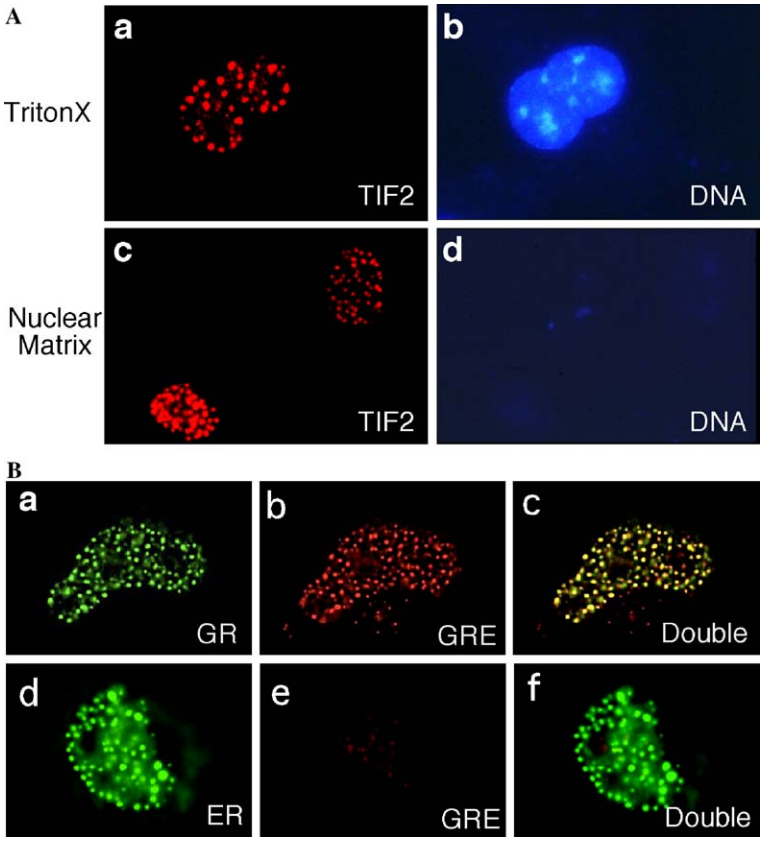

Fig. 3. (A) TIF2 protein is present in the nuclear structure even in the absence of intact chromatin DNA. Localization of TIF2 in COS-1 cells was examined after 0.5% Triton X-100 treatment (a,b) and in situ nuclear-matrix preparation (c,d) [40]. TIF2 was detected by using anti-Flag (M2) and Texas Red-conjugated secondary antibodies (a,c). DAPI staining was used to visualize chromatin DNA (b,d). (B) Recruitment of microinjected GREs to TIF2 foci via GR. The biotinylated GRE-containing oligo probes were injected into COS-1 cells, in which TIF2 was expressed simultaneously with GR-GFP (a–c) or ER-GFP (d–f). The cells injected with the probes were incubated with agonist ligand, 100 nM Dex for GR or 100 nM estradiol for ER. GRE probes were visualized with the anti-biotin and Texas Red-conjugated secondary antibodies (b,e). Specific colocalization of GR, but not ER, with the GRE probes was observed in TIF2 foci. These optical section images were analyzed on the computer-controlled, fluorescence microscope system described in Fig. 1.

matin DNA, occurs in the presence of ligand. They elegantly demonstrated that ligand-occupied GR was recruited to a specific chromatin region at which active transcription is occurring. Their results suggested that GR shuttles dynamically between its target sites on chromatin DNA and the matrix-associated TIF2 sites [17]. Furthermore, using chromatin immunoprecipitation (ChIP), Shang et al. [49] and Metivier et al. [50] showed that in response to estrogen, native ER transcription complexes were assembled in a sequential process on target promoters. These experiments demonstrated that the various coregulators of ER transcription were bound to ER after binding of ER to its target DNA. These data are also consistent with our observation that TIF2 exists as a preformed base for complex formation on the nuclear structure and that the ligand-bound nuclear hormone receptors move to the TIF2 complex with their target DNA [49,50]. Further understanding of the linkage between nuclear hormone receptor-mediated transcription and subnuclear compartments should be achieved by technical advancements allowing simultaneous visualization of nuclear receptors, coactivators, and their native DNA targets in a single living cell [51].

Acknowledgments

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