TR3 Orphan Nuclear Receptor Mediates Apoptosis through Up-regulating E2F1 in Human Prostate Cancer LNCaP Cells*

Xiaomin Mu and Chawnshang Chang‡

From the George Whipple Laboratory for Cancer Research, Departments of Pathology and Urology, University of Rochester Medical Center, Rochester, New York 14642

Early studies suggested both TR3 orphan receptor (TR3) and apoptosis mediator E2F1 might play an important role in mediating prostate cancer cell apoptosis. Their linkage and relationship, however, remain unclear. Here we found that 12-O-tetradecanoylphorbol-13-acetate (TPA) could induce cell apoptosis via induction of TR3 and E2F1 expression in LNCaP prostate cancer cells. Addition of antisense E2F1 could partially rescue the TR3-mediated cell apoptosis, and transfection of the TR3 dominant-negative plasmid could block the TR3-induced E2F1 expression. These data suggest that TPA is able to induce LNCaP cell apoptosis via induction of TR3 resulting in the induction of E2F1. Promoter reporter assays show that TR3 can induce E2F1 expression via binding to the TR3 response element (TR3RE) in the E2F1 promoter −316 to −324 bp region. TR3 can bind specifically to this TR3RE with a $K_d$ of 6.29 nM, and mutations of this E2F1-TR3RE can partially block the TR3-mediated E2F1 expression. Taken together, these data suggest that TPA is able to induce cell apoptosis via a TPA → TR3 → E2F1 → apoptosis pathway in LNCaP cells. Further studies of how to modulate this pathway may allow us to better understand how to control the prostate cancer growth.

Human TR3 orphan receptor, isolated by our laboratory (1), is the human homologue of mouse Nur-77 (2) and rat NGFIB (3, 4) genes. TR3 is initially classified as an immediate-early response gene and can be rapidly induced by diverse stimuli (2). TR3 is also involved in the regulation of apoptosis in various cell types (5–11). TR3 is rapidly induced during apoptosis of immature thymocytes and T-cell hybridomas and has been shown to be crucial in the T-cell receptor-mediated apoptosis in these cells (5, 6). TR3 has been shown to activate transcription through TR3 (NGFIB)-binding response element (TR3RE or NBRE) that was initially identified by genetic selection in yeast (12). The TR3RE contains the hexanucleotide AGGTCA, which is typically recognized by nuclear receptors of retinoic acid receptor/retinoid X receptor superfamily (13), and it includes two A residues preceding this hexanucleotide. Recognition of these two residues was shown to depend on TR3 sequences that lie outside the zinc finger domain (14). TR3 usually binds to TR3RE as a monomer (12, 14) and was also reported to activate pro-opiомelanocortin gene through another TR3 (Nur-77) response element, NurRE, as a homodimer (15). TR3 was also shown to form heterodimers with retinoid X receptor and to confer 9-cis-retinoic acid-dependent transcription to a reporter containing direct repeat (DR) 5 regulator element and TR3RE sequence (16).

The E2F family of transcription factors regulates expression of diverse mammalian genes for normal proliferation (17). Normal control of E2F activity is lost in many tumors, suggesting the central role of E2F in cell cycle control (18). In addition to the well established proliferative effect of E2F, one member of the family, E2F1, has been shown to mediate cell apoptosis (19). This property of E2F1 is unique because other family members of E2F are not believed to directly induce apoptosis (20). In the retinoblastoma protein-null mice, the apoptosis associated with loss of retinoblastoma protein function was shown to be efficiently rescued by deletion of E2F1, strongly supporting a role for E2F1 in apoptosis (21). E2F1 was later found to induce apoptosis in different cancer cell lines, and this is separable from the effects of this protein on cell cycle progression (22). Mice lacking E2F1 develop a broad unusual spectrum of tumors (23).

The proliferation of prostate cells and prostate cancer largely depends on the presence of androgen (24). The androgen effect is usually mediated by androgen receptors (25). Deprivation of androgens leads to increased cell death or apoptosis (26, 27). In the castrated rat prostate, many genes including c-myc, c-fos, c-jun, HSP70, and TGF-β have been reported to be up-regulated (27). However, little is known about the molecules and mechanisms that mediate apoptosis in the prostate. Although androgen deprivation has been a standard means of hormonal therapy for advanced prostate cancer patients, the molecular mechanism of androgen deprivation-induced prostatic apoptosis remains largely unknown. Both TR3 and E2F1 mediate cancer cell apoptosis (5–11); however, the linkage between them is unclear. We have reported previously that both the addition of androgen and the removal of androgen by castration induce expression of TR3 in the prostate and that antisense TR3 can increase LNCaP cell viability (10). Here we report that TR3 regulates the apoptosis by directly binding and up-regulating E2F1 in human prostate cancer LNCaP cells.

MATERIALS AND METHODS

Plasmids—TR3 expression plasmid pSG5-TR3 was constructed in our laboratory and reported previously (28). Doxycycline inducible pBG1 expression vector pBGI2i was described previously (29). The pBGI-E2F1 antisense fragment was cloned into the BamHI site of the pBGI2i vector. Dominant-negative TR3 (DN-TR3) expression plasmid was a gift from Dr. Whipple. Constructs of pE2F1a, pE2F1c, and pE2F1d were kindly provided by

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‡ To whom correspondence should be addressed. E-mail: chang@urmc.rochester.edu.

1 The abbreviations used are: TR3, human orphan receptor TR3; TPA, 12-O-tetradecanoylphorbol-13-acetate; TR3RE, TR3-binding response element; EMSA, electrophoretic mobility shift assay; DN, dominant-negative; Luc, luciferase; EGF, epithelial growth factor; DR, direct repeat.
Dr. Stephan Safe. pE2F1b and pE2F1a with TR3RE mutation (pE2F1b) were made by PCR.

Cell Culture and Transfections—Human prostate cancer PC-3 cells were maintained in Dulbecco’s modified Eagle’s medium containing penicillin (25 units/ml), streptomycin (25 μg/ml), and 5% fetal calf serum. Human prostate cancer LNCaP cells were maintained in RPMI 1640 containing the above concentrations of penicillin, streptomycin, and 10% fetal calf serum. PC-3 and LNCaP cells were transfected by transfection reagent (Qiagen, Chatsworth, CA).

Cell Viability—Cells were plated on 60-mm dishes and then the medium was changed after 24 h and was supplemented with 12-O-tetradecanoylphorbol-13-acetate (TPA) at varying concentrations. After TPA treatment, the cells were washed with PBS, trypsinized, collected, and resuspended in fresh medium, and viable cells were counted using 0.4% trypan blue staining.

Northern and Western Blot Analysis—Total RNA was isolated and Northern blot was performed as described previously (30). E2F1 antibody (KH95) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Immunoblot analysis was performed as previously described (30).

Electrophoretic Mobility Shift Assay (EMSA)—In vitro translation products were prepared using the T7T-coupled reticulocyte lysate system (Promega). EMSA was performed as described previously (28). The E2F1-TR3RE sequence and its complement were 32P-labeled and used for the probe. Oligonucleotides (sense strand) used for EMSA are E2F1-TR3RE, 5’-TATAGAAAGGTCAGTGGG-3’, and mutant E2F1-TR3RE, 5’-TATAGAAAGGCATGTCGTGGG-5’. Primers for TR3 are 5’-GACGCGTTACACAGAGAAG-3’, and 5’-AAGGCGCGGAGCAGAG-3’. Primers for E2F1 are 5’-AAGTCCACGACCCCACTGTCGTGGT-3’ and 5’-CCATACACCTCTCTGTCGTGG-3’. Primers for E2F3 are 5’-TCAACCACTACACAGACAGACG-3’ and 5’-CCGAGGCTGTCAGGAGGCACTG-3’. The results were normalized with β-actin.

Promoter Luciferase—For stable clones expressing antisense E2F1 and E2F1

Establishment of Stable Clones Expressing Antisense E2F1 and E2F1

Promoter Luciferase—For stable clones expressing antisense E2F1, LNCaP cells were plated on 100-cm dishes and were transiently trans-
fect with either 10 μg of plasmid or pcDNA3 vector. Clones resistant to hygromycin B (75 μg/ml) were isolated, expanded, and assayed for E2F1 expression after induction of doxycycline. For stable clones expressing E2F1 promoter luciferase, LNCaP cells were plated on 100-cm dishes and were transfected with a mixture (1:1 molar ratio) of E2F1–728 promoter reporter vector (pE2F-β) and pcDNA plasmid (Invitrogen) carrying a neomycin resistance gene (a total of 10 μg of DNA). Genetically resistant (300 μg/ml) clones were isolated, expanded, and assayed for luciferase activity.

**RESULTS**

**Induction of TR3 and E2F1 Gene Expression and Cell Death by TPA in Prostate Cancer LNCaP Cells—**Prostate cancer PC-3 cell growth is androgen-independent, and LNCaP cell growth is androgen-sensitive. We studied the effect of TPA on these two prostate cancer cell lines and studied molecules that mediate the TPA effect. We first found that TPA could strongly induce cell death in LNCaP cells (Fig. 1A, lane 4 versus lane 3) but not in PC-3 cells (Fig. 1A, lane 2 versus lane 1). These contrasting effects of TPA raised our interest to further examine cell death-related gene expression in these two cell lines. To our surprise, we found that both TR3 and transcription factor E2F1 were greatly induced in LNCaP cells but not in PC-3 cells (Fig. 1B). As a control, epithelial growth factor (EGF) has only a marginal effect on the induction of E2F1. These results are consistent with TPA differentially induced cell death in these two prostate cell lines. The EGF has been known to induce TR3 expression, yet fails to induce apoptosis in LNCaP cells (31). Combined with previous findings showing that both TR3 and E2F1 could mediate the apoptosis process in several cells and tissues (5–11), we hypothesized that TPA may be able to induce apoptosis in LNCaP cells via mediation of TR3 and E2F1.

**Dominant-negative of TR3 Can Abolish E2F1 Induction—**We have reported previously that antisense TR3 could increase prostate cancer cell viability in the presence of the apoptosis inducer etoposide (10). Other reports also showed that the inhibition of TR3 activity by expression of a DN-TR3 or its antisense RNA may result in the inhibition of apoptosis (5, 6, 9). Therefore, we studied the effect of DN-TR3 on E2F1 expression. As shown in Fig. 1C, TPA, which induces apoptosis in LNCaP cells, showed significant induction of E2F1 expression (Fig. 1C, lane 3 versus lane 1). Cotransfection of a plasmid-encoding DN-TR3 abolished this TPA-induced E2F1 expression (Fig. 1C, lane 6 versus lane 3). As controls, Fig. 1C also showed that EGF had only a marginal effect on E2F1 expression (lane 2 versus lane 1). Lane 1 was also used quantitative real-time RT-PCR to examine the effect of etoposide, another LNCaP cell apoptosis inducer, on the expression of E2F1 and E2F3 expression after TPA treatment. As shown in Fig. 1D, etoposide could also induce TR3 (lane 2 versus lane 1) and E2F1 expression (lane 4 versus lane 3), and the transfection of plasmid-encoding DN-TR3 partially abolished this etoposide-induced E2F1 expression (lane 5 versus lane 4). In contrast, the E2F3 mRNA level is not significantly changed after TPA treatment (Fig. 1E, lane 2 versus lane 1). Together, results from Fig. 1, C, D, and E suggest that TR3 may mediate TPA-induced cell death via E2F1 expression in LNCaP cells.

**Expression of E2F1 Antisense Can Reduce TPA-induced Cell Death in LNCaP Cells—**To further confirm that TPA-induced cell death is mediated by E2F1, we established a LNCaP cell line stably transfected with plasmid (pBIG-E2F1 antisense-LNCaP) and stably transfected LNCaP cells with pcDNA vector (pBIG-LNCaP) as a control. As shown in Fig. 2A, E2F1 expression was effectively inhibited by induction of E2F1 antisense expression using doxycycline in pBIG-E2F1 antisense-LNCaP cells, and the transfection of plasmid under the control of doxycycline (pBIG-E2F1 vector) and pcDNA plasmid (Invitrogen) carrying a neomycin resistance gene (a total of 10 μg of DNA). Genetically resistant (300 μg/ml) clones were isolated, expanded, and assayed for E2F1 expression after induction of doxycycline. For stable clones expressing E2F1 promoter luciferase, LNCaP cells were plated on 100-cm dishes and were transfected with a mixture (1:1 molar ratio) of E2F1–728 promoter reporter vector (pE2F-β) and pcDNA plasmid (Invitrogen) carrying a neomycin resistance gene (a total of 10 μg of DNA). Genetically resistant (300 μg/ml) clones were isolated, expanded, and assayed for luciferase activity.

**Fig. 2.** Expression of antisense E2F1 can partially rescue TPA-induced cell death in pBIG-E2F1 antisense-LNCaP cells. A, the suppression of E2F1 by the treatment of 6 μg/ml doxycycline (Dox) to induce E2F1 antisense expression in pBIG-E2F1 antisense-LNCaP cells is shown. B, E2F1 antisense-LNCaP cells and pBIG-LNCaP cells (lanes 4 and 2) were treated with 6 μg/ml doxycycline (lanes 2 and 4) and vehicle control (lanes 1 and 3) for 24 h. The cell lysates were collected and Western blot was performed. A representative blot from three independently performed experiments is shown. B, E2F1 antisense expression partially rescues TPA-induced cell death. pBIG-E2F1 antisense-LNCaP and pBIG-LNCaP cells were treated with control vehicle, 5 mM TPA, and 5 mM TPA plus 6 μg/ml doxycycline for 72 h, and viable cells were counted with trypan blue exclusion assay.
TR3 Orphan Nuclear Receptor Mediates Apoptosis in LNCaP

Fig. 3. The specific binding and higher binding affinity of TR3 to E2F1-TR3RE. A, binding of in vitro expressed TR3 to E2F1-TR3RE. EMSA was performed with the in vitro expressed TR3 and the 32P-labeled E2F1-TR3RE oligomers (lanes 1–10) or 32P-labeled mutant E2F1-TR3RE oligomer probes (lane 11). As negative controls, the binding reaction contained either no lysate (lane 1, probe only; lane 2, anti-TR3 antibody only) or lysate without TR3 (lane 3, probe and mock translated products). Binding reaction mixtures were incubated with the probe and in vitro expressed TR3 (lanes 4–11) in the presence of 10–100-fold (lane 6) excess of unlabeled E2F1-TR3RE as competitors or 10-fold (lane 7) or 100-fold (lane 8) excess unlabeled mutated E2F1-TR3RE as controls. Supershift of the TR3 protein-E2F1-TR3RE complex was induced in the presence of anti-TR3 antibody (lane 9) but not by anti-TR2 antibody (lane 10). When mutant E2F1-TR3RE was used as the probe, no probe-E2F1-TR3RE complex was formed (lane 11). The position of the TR3 protein-DNA complex and the supershift band are indicated by an arrow and arrowhead, respectively. B, the binding profile of in vitro expressed TR3 to various amounts of the probe. EMSA was performed with a constant amount of TR3 and varying concentrations of the labeled E2F1-TR3RE probe (0.0039–2 nM). The binding mixtures were resolved by 5% polyacrylamide gel. C, Scatchard analysis of TR3 affinity for E2F1-TR3RE. After autoradiography, the respective bands were excised, placed in scintillation fluid, and quantified directly in a scintillation counter. The ratio of activity between specific DNA-protein binding (Bound) and free DNA probe with respect to specific DNA-protein binding (Bound/Free) was plotted. The dissociation constant (Kd) and Bmax were determined from the minimum reciprocal of the slope of the line generated from the experimental data. The dissociation constant (Kd) of 6.29 nM and Bmax of 0.25 nM (Fig. 3C).

TR3 and TPA Up-regulates E2F1 Promoter Activity—Transient transfection in PC-3 and LNCaP cells of pE2F1a, a construct containing the −728/+77 region of E2F1 gene promoter linked to a luciferase (Luc) reporter gene, and TR3 expression plasmid pSG5-TR3 was then used to study potential roles of TR3 in regulating E2F1 promoter activity. As shown in Fig. 4, TR3 could up-regulate E2F1 gene expression in PC-3 (A) and LNCaP (B) cells in a dose-dependent manner (Fig. 4, A and B, lanes 2–4 versus lane 1). As a control, both androgen receptor and progesterone receptor (Fig. 4, A and B, lanes 6–8 versus lane 5) show little effect on the E2F1 promoter activity. We also transfected pE2F1a into LNCaP cells and treated the cells with 5 μM TPA. As shown in Fig. 4C, lane 2 versus lane 1, TPA could strongly induce E2F1 promoter activity. Furthermore, we established LNCaP cells stably transfected with pE2F1a-Luc (E2F1a-Luc-LNCaP). As shown in Fig. 4D, lane 2 versus lane 1, 5 μM TPA could strongly induce the Luc activity in E2F1a-Luc-LNCaP cells. Transfection of TR3 expression plasmid showed dose-dependent induction of Luc activity (Fig. 4E, lanes 2–4 versus lane 1) in E2F1a-Luc-LNCaP cells. As a control, the progesterone receptor (Fig. 4E, lanes 6–8 versus lane 5) shows little change in the Luc activity in E2F1a-Luc-LNCaP cells.

Mutation and Deletion of E2F1-TR3RE in the Human E2F1 Promoter Abolish the Up-regulation of E2F1 by TR3—To determine whether the up-regulation of E2F1 gene promoter activity by TR3 is through E2F1-TR3RE, we created pE2F1b by mutation of two nucleotides in the TR3RE of pE2F1a. These two nucleotide mutations resulted in the inability of TR3 to bind to E2F1-TR3RE in the EMSA assay (Fig. 3, lane 11). As shown in Fig. 5, the transient transfection in LNCaP (B) and PC-3 (C) cells of pE2F1b and pSG5-TR3 showed that the mutation of TR3RE partially abolished the up-regulation of E2F1 by TR3 (Fig. 5, B and C, lanes 6–8 versus lanes 2–4). We also used pE2F1c and pE2F1d, two E2F1 promoter reporter genes with the deletion of TR3RE, to determine that the up-regulation of E2F1 gene promoter activity by TR3 is through TR3RE. As shown in Fig. 5, the transient transfection in LNCaP (B) and PC-3 (C) cells of pE2F1c and pE2F1d showed that these two E2F1 gene promoters with deletion of TR3RE do not respond to TR3 (Fig. 5, B and C, lanes 10–12 versus lane 9 and lanes 14–16 versus lane 13). Furthermore, the induction of E2F1 promoter activity by TPA was greatly reduced by the replacement of wild type E2F1 promoter reporter pE2F1a with TR3RE mutated reporter pE2F1b (Fig. 5D, lane 2 versus lane 4). Altogether, the data from Fig. 5 suggested that the up-regulation of E2F1 promoter activity by TR3 was through E2F1-TR3RE.
FIG. 5. Deletion and mutation analysis of the E2F1 gene promoter for the promoter activity induced by TR3. A, a diagram of various TR3RE deletions and mutations in E2F1 promoter Luc reporter genes used in transient transfections. B and C, the mutation and deletion of TR3RE in E2F1 promoter significantly reduced or abolished E2F1 promoter activity induced by TR3. E2F1 promoter reporter genes shown in A were cotransfected with increasing amounts of pSG5-TR3 into LNCaP (B) and PC-3 cells (C). After 16–24 h, cells were collected and assayed for Luc activity. The results were normalized with an internal control (pRLSV40-Luc) and expressed as the mean ± S.E. of more than three independent experiments. D, the mutation of TR3RE reduced or abolished the TPA-induced E2F1 promoter activity. 5 µg of E2F1 promoter reporter genes 1a (lanes 1 and 2) or 1b (lanes 3 and 4) shown in A were transfected into LNCaP cells and the cells were treated with either vehicle control (lanes 1 and 3) or 5 mM TPA (lanes 2 and 4) for 4 h. Cells were collected and assayed for Luc activity. The results were normalized with an internal control (pRL-TK) and expressed as the mean ± S.E. of more than three independent experiments.

DISCUSSION

E2F1 gene expression is tightly regulated during the cell cycle. A slight overexpression of E2F1 could interfere with the cell cycle and induce extensive apoptosis. It has been known that E2F1 may go through both p53-dependent and p53-independent pathways to induce cell apoptosis. The p53-dependent pathway may involve the E2F1-mediated human tumor suppressor protein p14ARF, which neutralizes HDM2 (the human homologue of MDM2) and thereby stabilizes p53 protein (35). The p53-independent pathway involves induction of p73 by E2F1 (36). However, the factors that cause overexpression of E2F1 are largely unknown. TR3 is not expressed constitutively and is rapidly expressed only under certain kinds of stimulation. The central role of TR3 on T-cell receptor-mediated apoptosis in normal thymic-negative selection has been well established (5, 6). Interestingly, E2F1 has also been reported to mediate the apoptosis induced by T-cell receptor activation. E2F1-deficient mice have a thymocyte apoptosis defect (37), suggesting the existence of the cooperative effect between TR3 and E2F1.

Multiple SP1 sites, CCAAT motifs, and E2F1 binding sites have been identified in human E2F1 promoter (33, 34). Wang et al. (32) reported that E2 could activate E2F1 gene expression through estrogen receptor but failed to identify estrogen receptor response element in the promoter. We also observed strong expression of the E2F1 promoter by the estrogen receptor and E2F1 (data not shown). The identification of TR3RE in the E2F1 promoter could represent a new regulatory mechanism of E2F1 expression. We also analyzed the promoter sequences of other E2Fs, and we failed to find any TR3 sites in the promoter regions of other E2Fs, suggesting that TR3-modulated E2F1 may be selective among E2Fs.

Under normal conditions, E2F1 may mainly involve cell cycle regulation in cooperation with retinoblastoma protein and dimerization partner proteins. In some adverse conditions, TR3 is greatly and rapidly induced. TR3 could bind to E2F1-TR3RE in E2F1 promoter and enhance E2F1 expression. The overexpression of E2F1 could induce apoptosis through certain pathways. Thus, TR3-E2F1 constitutes a protective mechanism to push abnormal prostate cells into apoptosis and to prevent these cells from becoming cancer cells. As LNCaP cells are androgen-sensitive and less malignant, perhaps this TR3-E2F1 mechanism exists in LNCaP cells. PC-3 cells are androgen-independent and more malignant than LNCaP cells. Because TR3 cannot be induced in PC-3 cells, the TR3-E2F1 pathway does not exist in PC-3 cells. This could explain why TPA induces strong cell death in LNCaP cells but not in PC-3 cells.

In this study we used TPA as a potent apoptosis inducer in LNCaP cells. The other apoptosis inducers, such as calcium ionophore A23187, MM11453, or MM11364, which induce apoptosis in prostate cancer LNCaP cells, can also induce TR3 expression (31) suggesting the important role of TR3 in the apoptosis of LNCaP cells. It is possible that TPA-induced apoptosis is mediated by multiple pathways. This could explain why we only observed partial rescue of TPA-induced cell death by antisense E2F1 expression.

Most previous studies came to the conclusion that DNA binding and transactivation are required for the apoptotic effects of TR3 (5, 8, 12, 15). However, Li et al. (31) recently reported that TR3 might induce apoptosis through translocation from the nucleus to the mitochondria resulting in cytochrome c release and that transactivation of TR3 is not required for apoptosis. Katagiri et al. (38) also reported that nerve growth factor induced TR3 translocation from the nucleus to the cytoplasm. It is likely that TR3 mediates apoptosis through both transactivation-dependent and -independent pathways.

In summary, we found that TR3 mediated apoptosis through directly binding to E2F1-TR3RE and that it up-regulated E2F1 expression in human prostate LNCaP cells. Further studies of the TR3-E2F1-apoptosis pathway would assist us in learning how to control prostate cancer growth and progression.

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TR3 Orphan Nuclear Receptor Mediates Apoptosis in LNCaP

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