Activation of the β-Catenin/Lef-Tcf Pathway Is Obligate for Formation of Primitive Endoderm by Mouse F9 Totipotent Teratocarcinoma Cells in Response to Retinoic Acid*

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The morphogen retinoic acid promotes the formation of primitive endoderm in mouse F9 teratocarcinoma cells as does the stimulation of the Frizzled-1 pathway. We investigated whether the β-catenin/Lef-Tcf-sensitive transcriptional pathway activated by Frizzled-1 plays a role in the retinoic acid-induced pathway to primitive endoderm formation. An analysis of Lef-Tcf-sensitive transcription reveals increased transcription at 1 and 4 h post-treatment with retinoic acid. The stimulation of Lef-Tcf-sensitive transcription as well as the formation of primitive endoderm was accompanied by the stabilization of β-catenin as observed in activation of the Frizzled-1 pathway. Transient transfection of F9 cells with an expression vector harboring a dominant-negative mutant of Tcf4 resulted in the attenuation of both the increase in Lef-Tcf-sensitive transcription and formation of primitive endoderm in response to the morphogen. Clones stably transfected to express the dominant-negative Tcf4 displayed a block in retinoic acid-induced activation of Lef-Tcf-sensitive transcription and primitive endoderm formation. These data reveal the obligate role of the β-catenin/Lef-Tcf transcriptional pathway in the action of the morphogen retinoic acid.

Retinoic acid is a well known morphogen involved in many aspects of mammalian development (1). Our understanding of the mechanism(s) by which retinoic acid functions is quite detailed at the level of nuclear receptors controlling gene expression for this morphogen (2, 3). Retinoids like retinoic acid act via the retinoic acid receptor and retinoid receptor proteins, which modulate gene expression through binding to retinoic acid-responsive elements of target genes. Another class of morphogens, Wnts, is a class of vertebrate genes encoding secreted signaling proteins that appear to modulate diverse processes in enabling retinoic acid to promote the formation of primitive endoderm (27, 31–35). These cells, retinoic acid acts in opposition to the β-catenin-Tcf-Lef pathways. In these cells, retinoic acid promotes the formation of primitive endoderm by mammalian multipotent teratocarcinoma cells in culture. Cross-regulation between retinoid signaling and the Wnt-1 via Frizzled homologues activates the function of Dvl, which represses the activity of glycogen synthase kinase 3 (6, 25), promoting the elevation of intracellular β-catenin levels and accumulation of β-catenin in nuclei (14, 26). Nuclear β-catenin interacts with members of the T-cell factor/lymphoid enhancer-binding factor (Tcf-Lef) classes of architectural high mobility group box transcription factors (27–30) to regulate the expression of genes involved in vertebrate development (27, 31–35).

Recently, we demonstrated that the activation of the Frizzled-1 receptor expressed in mouse F9 teratocarcinoma cells like retinoic acid promotes the formation of primitive endoderm (36). The Frizzled-1 receptor signals via heterotrimeric G-proteins to the β-catenin-Tcf-Lef-sensitive transcriptional pathway (37). Studies with the native rat Frizzled-1 receptor (36) as well as with a novel chimeric receptor employing the cytoplasmic domains of Frizzled-1 with the transmembrane and exofacial regions of the β2-adrenergic receptor (37) demonstrate the stabilization and accumulation of β-catenin, the activation of Tcf-Lef-sensitive transcription, and the formation of primitive endoderm. Retinoic acid stimulates the formation of primitive endoderm in mammalian stem cells (38, 39). Cross-regulation between retinoid signaling and the β-catenin-Tcf-Lef pathways has been reported in MCF-7 breast cancer cells in culture (40). In these cells, retinoic acid acts in opposition to the β-catenin-Tcf-Lef pathway. In the current work, we investigate a novel hypothesis that the β-catenin/Lef-Tcf pathway participates in the ability of retinoic acid to promote the formation of primitive endoderm much like it does in Wnt-Frizzled-1 signaling. The results demonstrate a new role for β-catenin-Tcf-Lef signaling in enabling retinoic acid to promote the formation of primitive endoderm by mammalian multipotent teratocarcinoma cells in culture.

EXPERIMENTAL PROCEDURES

F9 Cell Culture and Transfection Studies—Mouse F9 teratocarcinoma cells were obtained from the ATCC collection, propagated, and stably transfected using LipofectAMINE (Invitrogen) with the pcDNA3.0 vector engineered by standard techniques to express the Tcf-Lef-sensitive luciferase reporter gene TOPFLASH (32) or the dominant-negative interfering mutant form of Tcf4 (Tcf4-DN) (41) under the control of the cytomegalovirus promoter (42, 43). The expression vector for the dom-

* This work was supported in part by grants from the National Institutes of Health (to C. C. M.) and by an award from the March-of-Dimes Foundation (to H. Y. W.). 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.

¶ Recipient of an NRSA postdoctoral fellowship 5T32 DK07521 from the NIDDK, National Institutes of Health.

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The abbreviations used are: Tcf-Lef, T-cell factor-lushyp; enhancer-binding factor; Tcf4-DN, dominant-negative mutant form of Tcf4; RT, reverse transcription; TROMA, antibody to cytoherin endoA; TPA, tissue plasminogen activator.
Activation of β-Catenin/Lef-Tcf Pathway by Retinoic Acid

Inant-negative Tcf4 was generously supplied by Dr. Eric R. Fearon (Division of Molecular Medicine & Genetics, University of Michigan Medical Center, Ann Arbor, MI). The vector harbors a copy of the neomycin-resistant gene, and Tcf4-DN(ΔN31) clones were selected in medium containing the neomycin analogue G418 (0.4 mg/ml, Invitrogen). 10–20 independent clones resistant to the G418 were propagated. The level of expression of the mRNA for Tcf4-DN was measured indirectly via reverse transcription polymerase chain reaction amplification using primers 5′-ACTACAAAGGCAAGCAGTACAAG-3′ and 5′-ACGTGATACAGGCGCTGGAGG-3′. The clones displaying the highest level of expression of mRNA for dominant-negative Tcf4 were then employed for the studies reported herein. Controls for the reverse transcription and PCR amplification (RT-PCR) were provided using primers for RNA of cyclophilin A, a non-target for retinoic acid induction.

Analysis of β-catenin Stabilization and Transcriptional Activation by Tcf-Lef Proteins—For the study of β-catenin stabilization, F9 cells were pretreated with and without cycloheximide and then treated with 50 nM retinoic acid for 0–6 h, and the cytoplastic β-catenin was extracted in the supernatants of whole cell lysates treated with ConA-Sepharose to remove cadherin-associated β-catenin. Aliquots of the cell supernatants were subjected to SDS-PAGE, and the resolved protein transferred to blots. Immunoblots of β-catenin were stained with anti-β-catenin antibodies (C2206, Sigma) and detected by ECL, and the amount of stain was quantified by imaging densitometry on a Bio-Rad GS-700. F9 wild-type cells and clones stably transfected to express dominant-negative Tcf4 were propagated and then transiently transfected either with plTOPFLASH plasmid, which contains three copies of the optimal Tcf motif CCCTTGATC upstream of a minimal c-Fos promoter driving luciferase expression, or the pFOFLASH plasmid that contains a mutated motif CCCTTGCCTC of the Tcf-Lef-sensitive elements. The TOPFLASH controls reveal no expression of luciferase in the absence or presence of retinoic acid (37). An additional β-catenin-sensitive reporter (cyclin D1 promoter luciferase reporter gene) was employed as a further control. The cyclin D1 promoter-driven reporter gene was a gift from Dr. Randy Moon (Howard Hughes Medical Institute and the Department of Pharmacology, University of Washington, Seattle, WA). The clones were co-transfected with a second plasmid that expresses the gene for β-galactosidase, which was used to measure the efficiency of the transfection. Clones were challenged with 50 nM retinoic acid, and the activation of Tcf-Lef-sensitive luciferase reporter gene was determined.

RESULTS AND DISCUSSION

The activation of Frizzled-1 receptors in mouse F9 cells stimulates the activation of Lef-Tcf transcription and the formation of primitive endoderm via the stabilization of β-catenin (36, 37). Using the TOPFLASH luciferase reporter gene that reflects the activation of Lef-Tcf-sensitive transcription, we examined whether treatment with the morphogen retinoic acid altered the β-catenin-Lef pathway (Fig. 1a). Wild-type F9 cells were treated with retinoic acid (+RA), and the activation of TOPFLASH was monitored over the next 9–10 h. There was a generalized increase in the transcription of the Lef-Tcf-sensitive reporter gene over the entire 10 h following treatment with retinoic acid, and two periods of significantly greater activity were noted at 0.5 and 4 h post-retinoic acid. At the 4-hour time point, the level of transcription was 4–5-fold greater in the presence of retinoic acid.

Three controls were conducted to further analyze the response. We stably transfected Lef-Tcf-sensitive transcription independent of retinoic acid using the activation of the rat Frizzled-1 (Rfz1) chimera to mimic the Wnt-8/Frizzled-1 signaling (37). F9 clones were transiently transfected with the β-adrenergic receptor/Rfz1 and stimulated (Fig. 1b). Although the time course for Frizzled-1 activation of TOPFLASH was different from that observed for retinoic acid, the magnitude of the two responses was comparable. As a control, F9 clones were transiently transfected with FOPFLASH, a luciferase reporter construct that lacks the β-catenin binding sites. FOPFLASH activity in response to stimulation of the Frizzled-1 pathway (Fig. 1b) or retinoic acid (data not shown) was unaltered from the basal levels. Finally, we also tested the response of an endogenous luciferase reporter sensitive to β-catenin, the cyclin D1-luciferase reporter gene, and observed the same response (Fig. 1c) and noted in assays using the TOPFLASH luciferase reporter gene (Fig. 1a).

Because the activation of Lef-Tcf-sensitive transcription should reflect the stabilization of β-catenin, we assayed the levels of free β-catenin in F9 cells following stimulation with retinoic acid (Fig. 1d). The stabilization of β-catenin levels was evident in response to retinoic acid. The levels of β-catenin increased some 7-fold within 4 h of treatment with the morphogen. The time course for stabilization of β-catenin correlates well with the changes observed in the transcriptional activation of the Lef-Tcf-sensitive reporter genes TOPFLASH and cyclin D1 (Fig. 1, a and c). To determine whether the increase in β-catenin was solely the result of post-translational stabilization, we performed the analysis of accumulation of free β-catenin in cells treated in advance with the protein synthesis inhibitor cycloheximide (Fig. 1e). Cycloheximide treatment failed to abolish the accumulation of free β-catenin, an observation consistent with post-translational stabilization of β-catenin in response to retinoic acid. The character of the time course was not altered, although the magnitude of the response was diminished.

The ability of retinoic acid to stabilize the accumulation of β-catenin and to activate the TOPFLASH reporter gene was a significant insight into the important question of the role of the β-catenin-Tcf-Lef pathway in retinoic acid action. Although these observations provide compelling data in support of a rather unique role of the β-catenin-Tcf-Lef pathway in the activation of this morphogen, we sought to test this linkage further with the aid of dominant-interfering mutants of Tcf. The Tcf4-DN lacks the binding sites for β-catenin binding and activation (41). The expression of this construct has been shown to block the β-catenin-Lef pathway of transcriptional activation (41). Initial experiments were performed using transient transfection to introduce the expression of Tcf4-DN. F9 cells were transiently transfected and examined at 24 h post-infection for the ability of retinoic acid to activate the TOPFLASH reporter gene (Fig. 2a). Wild-type cells transiently transfected with the empty vector displayed a 2–3-fold increase in the transcription of TOPFLASH. Transient expression of the Tcf4-DN resulted in little change in the amount of background transcription of TOPFLASH but blocked completely the ability of retinoic acid to activate TOPFLASH.

Secretion of tissue plasminogen activator (tPA) is a hallmark of primitive endoderm formation and begins within the first 2–3 days following treatment with retinoic acid (38). We tested the effects of transient transfection of Tcf4-DN on the response of the F9 cells treated with retinoic acid and then monitored for secretion of tPA 2 days later (Fig. 2b). Although not as robust as the tPA response at 4–6 days, the secretion of tPA was stimulated ~6-fold in F9 cells treated with retinoic acid for 2 days (39). In those cells transiently transfected to express the Tcf4-DN, the basal level of tPA secretion was normal, but the tPA secretion in response to morphogen was reduced by at least half. These observations suggest that the β-catenin-Tcf-Lef pathway is important but perhaps not obligate for the retinoic acid-induced formation of primitive endoderm from these totipotent teratocarcinoma cells. These are the first indications that there is some cross-talk between the well known retinoic acid-stimulated pathway to primitive endoderm formation and
To complete the analysis of the role of Tcf-Lef-sensitive transcription in retinoic acid action on F9 cells, we adopted a different approach of creating stable transfectant clones expressing the Tcf4-DN (Fig. 3). F9 clones were stably transfected with pcDNA3.0 harboring the cDNA for Tcf4-DN and placed under a selectable marker, neomycin-resistant. The resultant clones were screened for the expression of Tcf4-DN mRNA using specific primers in combination with RT-PCR. The amplification products of clones were separated on agarose gels, and positive clones were identified by the appearance of a 476 bp product (Fig. 3a).

We next examined both the stable transfectants expressing Tcf4-DN as well as those transfected with empty vector and measured the activation of TOPFLASH in response to retinoic acid (Fig. 3b). In wild-type F9 cells as well as in the clones stably transfected with empty vector (Fig. 3a, b), similar experiments were performed in F9 cells transiently transfected with the β2-adrenergic receptor/βfz1 chimera, stimulated with 10 μM isoproterenol, and then assayed using the TOPFLASH luciferase reporter gene. As a control, the same experiments were performed with the FOPFLASH luciferase reporter gene that lacks the β-catenin binding sites as shown in b. FOPFLASH luciferase activity was not increased above basal level in response to the activation of the Wnt-Frizzled-1 pathway using the chimera. The ability of retinoic acid to stimulate transcription of the cyclin D1 promoter-driven luciferase reporter gene was also examined (c) in the same manner as employed for TOPFLASH (a). d and e, intracellular levels of β-catenin also were measured in response to retinoic acid. The data shown in a–c are the means ± S.E. of 3–5 separate experiments and are reported in relative light units (RLU). For study of the contribution of new protein synthesis to the accumulation of β-catenin, cells were treated for 1 h in advance and also during the treatment with retinoic acid with 50 μM cycloheximide to block protein synthesis (e). The stability of β-catenin as shown in d and e was assessed by SDS-PAGE of the intracellular complement of β-catenin. The proteins were transferred to blots, stained with antibodies to β-catenin, and the amount of stain was quantified.

**Fig. 1. Retinoic acid treatment stimulates β-catenin accumulation and Tcf-Lef-sensitive transcription.** Wild-type F9 teratocarcinoma cells were treated with 50 nM retinoic acid (+RA) at t = 0. a, over the next 10 h, the activation of the Tcf-Lef-sensitive luciferase reporter gene TOPFLASH was monitored. b, similar experiments were performed in F9 cells transiently transfected with the β2-adrenergic receptor/βfz1 chimera, stimulated with 10 μM isoproterenol, and then assayed using the TOPFLASH luciferase reporter gene. As a control, the same experiments were performed with the FOPFLASH luciferase reporter gene that lacks the β-catenin binding sites as shown in b. FOPFLASH luciferase activity was not increased above basal level in response to the activation of the Wnt-Frizzled-1 pathway using the chimera. The ability of retinoic acid to stimulate transcription of the cyclin D1 promoter-driven luciferase reporter gene was also examined (c) in the same manner as employed for TOPFLASH (a). d and e, intracellular levels of β-catenin also were measured in response to retinoic acid. The data shown in a–c are the means ± S.E. of 3–5 separate experiments and are reported in relative light units (RLU). For study of the contribution of new protein synthesis to the accumulation of β-catenin, cells were treated for 1 h in advance and also during the treatment with retinoic acid with 50 μM cycloheximide to block protein synthesis (e). The stability of β-catenin as shown in d and e was assessed by SDS-PAGE of the intracellular complement of β-catenin. The proteins were transferred to blots, stained with antibodies to β-catenin, and the amount of stain was quantified.
lanes 1 and 3), the basal levels of TOPFLASH activation and those in response to retinoic acid were comparable (Fig. 3b, bar graphs for WT and clones 1 and 3). In sharp contrast, the clones expressing Tcf4-DN mRNA (Fig. 3a, lanes for clones 2 and 4) were found to show little activation of the TOPFLASH reporter gene (Fig. 3b, bar graphs for clones 2 and 4). These data demonstrate that with adequate levels of stable expression of the Tcf4-DN, retinoic acid-induced changes in Tcf-Lef-sensitive transcription are completely blocked.

We tested further the role of the β-catenin-Tcf-Lef pathway in the F9 cell clones expressing the Tcf4-DN by examination of the morphology as well as for the expression of the TROMA antigen, a hallmark of primitive endoderm (38). Wild-type F9 cells and the clones expressing the Tcf4-DN were stimulated with retinoic acid and then examined 4 days later by immunohistochemical techniques (Fig. 3c). In the wild-type F9 cells, retinoic acid leads to a pronounced change in cell morphology as observed by phase-contrast microscopy. Immunohistochemical staining and epifluorescence microscopy of the cells with the monoclonal TROMA antibodies to endo-A revealed universal staining of the cultures at 4 days after treatment with morphogen. The clones expressing the Tcf4-DN in sharp contrast to the wild-type cells displayed no change in cellular morphology and little positive staining for the TROMA antigen 4 days after treatment with morphogen. These data specifically those on β-catenin stabilization and on the activation of the TOPFLASH reporter gene provide compelling evidence that the β-catenin-Tcf-Lef pathway provides an indispensable signal in the morphogenic action of retinoic acid.
Retinoic acid is a well known morphogen that acts via nuclear receptors to exert its effects on vertebrate development (3). Recently, we observed that the activation of rat Frizzled-1 receptor in F9 cells was accompanied by the formation of primitive endoderm via a mechanism that requires heterotrimeric G-protein signaling and the stabilization of free β-catenin levels (37). We have demonstrated that Frizzled receptors indeed couple to heterotrimeric G-proteins and are members of the superfamily of G-protein-coupled receptors (44). The increase in β-catenin levels leads to the activation of Tcf-Lef-sensitive transcription. The similarities in the effects of Wnts and retinoic acid (i.e. stimulation of F9 teratocarcinoma cells to form primitive endoderm) prompted us to investigate whether or not the β-catenin/Lef pathway was playing some role in the action of retinoic acid. Earlier, it was reported in MCF-7 breast cancer cells in culture that retinoic acid decreases the activity of the unstimulated β-catenin-Tcf-Lef signaling pathway (40). This observation prompted us to explore the role of retinoid action in β-catenin-Tcf-Lef signaling in the F9 cells. In this work, we demonstrate that the treatment with retinoic acid, in fact, does stimulate the stabilization of β-catenin levels. Furthermore, we demonstrated using the TOPFLASH luciferase gene reporter that the treatment with retinoic acid prompts an activation of Lef-Tcf-sensitive transcription. The expression of the dominant-negative Tcf4 abolished the ability of retinoic acid to promote the formation of primitive endoderm. These data are the first to reveal a synergistic cross-talk between the actions of retinoic acid and the β-catenin-Tcf-Lef pathways. The differences observed between the F9 totipotent teratocarcinoma cells and the MCF-7 cells with regard to the role of β-catenin-Tcf-Lef signaling highlight perhaps differences in the cellular context and the roles of retinoid acid. Further studies will be directed at establishing the mechanism by which retinoic acid stabilizes β-catenin levels, a mechanism of gene activation shared by retinoic acid and Wnt ligands.

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J. Biol. Chem. 2002, 277:30887-30891.
doi: 10.1074/jbc.M203852200 originally published online June 12, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M203852200

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