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Induction of Differentiation in F9 Cells and Activation of Peroxisome Proliferator-Activated Receptor δ by Valproic Acid and Its Teratogenic Derivatives

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ABSTRACT

The antiepileptic drug valproic acid (VPA) is teratogenic, because it induces birth defects in some children of mothers treated for epilepsy. Cellular and molecular actions associated with teratogenicity were identified by testing differentiation of F9 embryocarcinoma cells. VPA altered cell morphology and delayed proliferation. Specific differentiation markers (e.g., c-fos and keratin 18 mRNA and particularly the activating protein-2 transcription factor protein) were induced. This pattern differs from the pattern induced by other teratogens or F9 cell-differentiating agents. Induction of differentiation correlated with teratogenicity because teratogenic derivatives of VPA, such as (*S*)-4-yn-VPA, induced differentiation, whereas closely related nonteratogenic compounds, such as (*R*)-4-yn-VPA, 2-en-VPA, and 4-methyl-VPA, did not. In the cellular signaling network, the peroxisome proliferator-activated receptor δ (PPARδ) was activated selectively by VPA and teratogenic derivatives. Depletion of PPARδ by antisense RNA expression precluded the response of F9 cells to VPA. In conclusion, our data show that VPA and its teratogenic derivatives induce a specific type of F9 cell differentiation and that PPARδ is a limiting factor in the control of differentiation.

The antiepileptic drug valproic acid (VPA; 2-propyl-pentanoic acid) is a potent teratogen in both human and mouse. Epileptic women treated with this drug give birth to children with a risk of about 1 to 50 of having a defect in the closure of the neural tube (e.g., spina bifida occulta or aperta). Additional alterations that are collectively called the embryonic valproate syndrome include malformations of the facial skull and the heart (DiLiberti et al., 1984; Huot et al., 1987; Ardinger et al., 1988; Martinez-Frias, 1990, 1991). VPA treatment of pregnant mice induces litters with most of the embryos showing an incomplete neural tube closure (Nau et al., 1991). The type of defect depends on the time of application. Treatment at an embryonic age of 8.25 days after conception induces exencephaly, a closure defect of the anterior part of the neural tube, including malformations of the brain. Repeated treatments between the embryonic age of 9 and 9.5 induce closure defects of the posterior part of the neural tube, which become apparent at later stages as spina bifida.

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It is likely that VPA acts on preexisting signaling pathways and cellular programs that are required for proper embryonic development around the time of neural tube closure. The existence of a specific interaction of VPA with cellular signaling events is supported by the finding that VPA teratogenicity in the mouse is subjected to stringent structure-activity constraints. Thus, introduction of a double bond between carbon 2 and 3 (2-en-VPA) renders the derivative nonteratogenic, although still antiepileptic. A triple bond between carbon 4 and 5 (4-yn-VPA) generates a derivative that is highly teratogenic, but relatively poorly antiepileptic. Teratogenicity of 4-yn-VPA is selective for the enantiomer applied; e.g., (*S*)-4-yn-VPA is highly teratogenic, whereas (*R*)-4-yn-VPA is not. Both forms do not differ with respect to their antiepileptic activity. An additional substitution in the second branch of the molecule, 4-yn,4′-Me-VPA renders the compound nonteratogenic (Nau et al., 1991; Hauck et al., 1992). The teratogenic compounds in this series of compounds are expected to affect either proliferation, differentiation, or function of cells during the sensitive time period of embryonic development. Teratocarcinoma cells, such as P19 or F9, have properties similar to those of early embryonic cells. Appropriate stimuli, such as retinoids, cAMP-signaling, growth factors, or lack of adhesion induce

ABBREVIATIONS: VPA, valproic acid; PPAR, peroxisome proliferator activated receptor; GR, glucocorticoid receptor; RA, retinoic acid; AP, alkaline phosphatase; AP-2, activating protein-2; NCC, neural crest cell; EMSA, electrophoretic gel mobility shift analysis ("band-shift").

them to differentiate. Depending on the stimulus differentiated F9 cells show properties and marker gene expression of either endodermal, ectodermal, or mesodermal cells (Kellermann et al., 1987; Lehtonen et al., 1989). Indirect evidences suggest that also VPA could alter the properties of F9 cells (Lampen et al., 1999).

The goal of the present study was to identify conditions of VPA-induced differentiation, which are clearly defined by cellular and biochemical parameters. A model system of differentiation should be established that faithfully reflects the structure-activity relationship of teratogenicity among VPA derivatives. Furthermore, VPA-sensitive cellular signaling molecules were to be identified and their role in the VPA-dependent cellular responses clarified.

Using F9 (and P19) teratocarcinoma cells we demonstrated that VPA induces a specific type of cell differentiation, which differs from that induced by other established inducers of F9 cell differentiation. The search for a VPA-responsive "receptor" molecule was guided by the observation that VPA treatment of rodents induces proliferation of peroxisomes in liver cells (Horie and Suga, 1985; Ponchaut et al., 1991) and the chemical structure of VPA (e.g., a carboxylic acid). Induction of peroxisomal proliferation is mediated by a subgroup of the steroid receptor superfamily, the peroxisome proliferator-activated receptors (PPARs) (Forman et al., 1996; Willson and Wahli, 1997). Three forms are known, PPARα, PPARγ, and PPARδ, the latter of which is also called PPARβ, FAAR, or NUC1. They have distinct expression patterns, are activated by various carboxylic acids, peroxisome proliferation-inducing drugs, or eicosanoids, and fulfil different nonredundant physiological functions (Issemann and Green, 1990; Göttlicher, 1992; Kliewer et al., 1994; Tontonoz et al., 1994; Amri et al., 1995), among which the role of PPARδ is understood least.

We now extend previous data from a hybrid receptor approach (Lampen et al., 1999) by showing that VPA activates PPARδ-dependent transcription also in the context of the native receptor. More importantly, stable expression of PPARδ antisense RNA in F9 cells provides evidence for the fact that PPARδ indeed is a limiting factor in the regulation of F9 cell differentiation rather than merely a surrogate marker for VPA-induced alterations in cell function.

Materials and Methods

Cell Culture and Drug Treatment. Culture of Chinese hamster ovary cells, stable transfection, and detection of the alkaline phosphatase reporter gene were performed as described previously (Göttlicher et al., 1992). F9 cells (American Type Culture Collection, Manassas, VA) were cultured on dishes precoated with 0.1% gelatin in PBS. The culture medium was Dulbecco's modified Eagle's medium/Ham's F-12 (1:1) supplemented with 2 mM glutamine, 0.15 mM β-mercaptoethanol, and 10% fetal bovine serum. VPA and derivatives, except for 2-en-VPA, were dissolved as liquids in the cell culture medium. 2-en-VPA and retinoic acid (RA) were added as solutions in dimethyl sulfoxide (1 M and 100 mM stock solutions, respectively). Sodium butyrate and dibutyryl-cyclic AMP were dissolved in aqueous media. Staining the F-actin cytoskeleton with BODIPYFL phallacidin followed the procedures recommended by the supplier (Molecular Probes, Eugene, OR).

Plasmid Construction. The expression vector for GR-PPARδ was constructed by releasing the ligand-binding domain of PPARα from pMT-GR-PPARα (Göttlicher et al., 1992) by cleavage with *Xba*I (3′) and Klenow fill-in followed by *Xho*I digestion (5′). The cDNA for the ligand-binding domain of PPARδ was prepared from the Gal4-PPARδ expression vector (Kliewer et al., 1994) as *Kpn*I (Klenow-blunted)-*Bam*HI fragment and subcloned into pGem7Zf prepared by *Kpn*I (Klenow-blunted)-*Bam*HI digestion. The fragment was recovered as a *Xho*I-*Bam*HI (Klenow-blunted) fragment containing the vector-derived sequence tcgaGGAATTC as adaptor in the 5′ end and cloned into the pMT-GR-vector prepared as described above.

The PPARδ-responsive reporter gene PDRE4-Mluc was constructed by subcloning a tetramer of a PPARδ-responsive element as *Nhe*I (partially filled with Klenow polymerase) *Eco*RV fragment from p4xDRE-Luc (He et al., 1999) into pMLuc prepared by digestion with *Hin*dIII/*Nhe*I (partially filled with Klenow polymerase) and *Sma*I.

PPARδ antisense RNA expressing F9 cells were generated first by stably transfecting an expression vector for the tet *trans*-activator (pTet-Off; CLONTECH, Palo Alto, CA), thus generating the F9^{tetoff} subclone. The major part of the PPARδ cDNA was cloned as a *Bam*HI (blunted by Klenow fill in)-*Xba*I fragment into pBI-L (CLONTECH) prepared by cleavage with *Nhe*I and *Eco*RV. This vector, called pBI-aPPARδ, was supposed to express both luciferase and PPARδ antisense RNA under control of the same tet *trans*-activator binding site. pBI-aPPARδ was stably transfected into F9^{tetoff} cells.

RNA and Protein Analysis. Poly(A)$^+$ RNA preparation and Northern blot analysis followed standard procedures. Probes for PPAR mRNA detection were fragments comprising nucleotides 378 to 519 of the rat PPARα cDNA (Göttlicher et al., 1992), corresponding to the amino acids 340 to 456 of PPARγ2 (Tontonoz et al., 1994) and the 200-base pair *Pst*I fragment of PPARδ (FAAR) covering the translational start site (nucleotides 31–230) (Amri et al., 1995). AP-2 protein expression was detected by Western blot analysis of F9 cell nuclear extracts using a rabbit polyclonal antibody raised against an AP-2α peptide (Santa Cruz Biotechnology, Santa Cruz, CA). For nuclear extract preparation F9 cells were harvested by incubation in PBS without Ca^{2+} or Mg^{2+} containing 5 mM EDTA. Cell pellets were resuspended and lysed in a hypotonic buffer (25 mM Tris pH 7.6, 1 mM EDTA) containing 0.05% NP-40 for 20 min on ice. Nuclei were collected by centrifugation and subjected to lysis in a sample buffer for SDS acrylamide gel electrophoresis.

Transient Transfections. F9 cells were transfected in six-well culture dishes for 4 h by the calcium phosphate coprecipitation method. Transfection mixes contained 1 μg of the PDRE4-Mluc reporter gene together with 0.1 μg of renilla luciferase controlled by the ubiquitin C promoter for normalization and, if applicable, 0.2 μg of expression vectors for RXR and PPARδ (Amri et al., 1995). Cells were treated for 17 to 20 h with 1 mM VPA before the analysis of reporter gene activity.

Gel Mobility Shift Analysis. Nuclear protein extracts from appropriately treated F9 cells were prepared by standard mild detergent lysis of cells (0.05% NP-40) and high salt extraction of nuclear proteins (20 mM Hepes, pH 7.9; 0.2 mM EDTA; 0.5 mM dithiothreitol; 1.5 mM $MgCl_2$; 420 mM NaCl; 25% glycerol; 0.5 mM phenylmethylsulfonyl fluoride). The salt concentration was reduced to a final concentration of 75 mM NaCl by dilution. Twenty-microliter bandshift reactions with 5 μg of nuclear protein were performed in a buffer (62 mM Tris-HCl, pH 7.8; 0.6 mM EDTA; 5 mM dithiothreitol; 75 mM NaCl; 6% glycerol) containing 2 μg of poly(dIdC) (Pharmacia, Freiburg, Germany) and 10 fmol of a ^{32}P-labeled probe. If appropriate antibodies or nonlabeled oligonucleotides were added, preincubation for 15 min on ice was followed by the addition of 0.1 pmol of the labeled probe. After 15 min at room temperature, samples were separated on a 5% acrylamide gel in 0.5× Tris borate buffer. The following oligonucleotides were used: PPARδ-RE: CTAGCGTGAGCGCTCACAGGTCAATTCG and CTAGCGAATTGACCTGTGAGCGCTCACG; and AP-2-RE: TCGAACTGACCGCCCGCGGCCCGTGTGC and TCGAGCACACGGGCCGCGGGCGGTCAGT.

RNA in Situ Hybridization. RNA in situ hybridization was performed in accordance with standard procedures using a ^{35}S-labeled probe, which comprised 57 nucleotides of the 5′ untranslated

region and the first 1137 nucleotides of the FAAR open reading frame (Amri et al., 1995).

Results

Differentiation of F9 Cells by VPA. Induction of F9 cell differentiation by VPA was analyzed using the criteria of altered cell morphology, reduced proliferation, and expression of marker genes. VPA treatment for 2 days induced profound changes in cell morphology, which were characterized by less tightly packed cells within the colonies and the generation of long filamentous structures. The latter were detected by staining of the F-actin cytoskeleton (Fig. 1). Cell proliferation was reduced by VPA, as reflected by reduced [^{3}H]thymidine incorporation and cell recovery (Table 1).

Differentiation of F9 cells to different cell types by chemicals, such as RA, cAMP or dimethyl sulfoxide, is characterized by the expression of distinct marker genes (for review, see Lehtonen, et al., 1989; Alonso et al., 1991). The effect of VPA on such marker genes was analyzed in comparison to the differentiating agent RA (Strickland and Mahdavi, 1978; Solter et al., 1979). Expression of the *keratin 18* gene was clearly induced (3-fold) by VPA and marginally (1.7-fold) by RA. *Laminin β1* and *collagen (a1)IV* were induced by RA, but not by VPA (Fig. 2A; Table 2). Also, the *c-fos* gene, which is associated with and capable of inducing F9 cell differentiation (Müller and Wagner, 1984), was inducible by VPA (Fig. 2B). The kinetics of *c-fos* induction by VPA differed from the immediate early response to phorbol esters. The latter was rapidly terminated and mRNA levels had reached control levels within 9 h (data not shown), whereas VPA-induced *c-fos* expression persisted (Fig. 2B). The observation time in this experiment was most likely too short to find *c-fos* induction by RA.

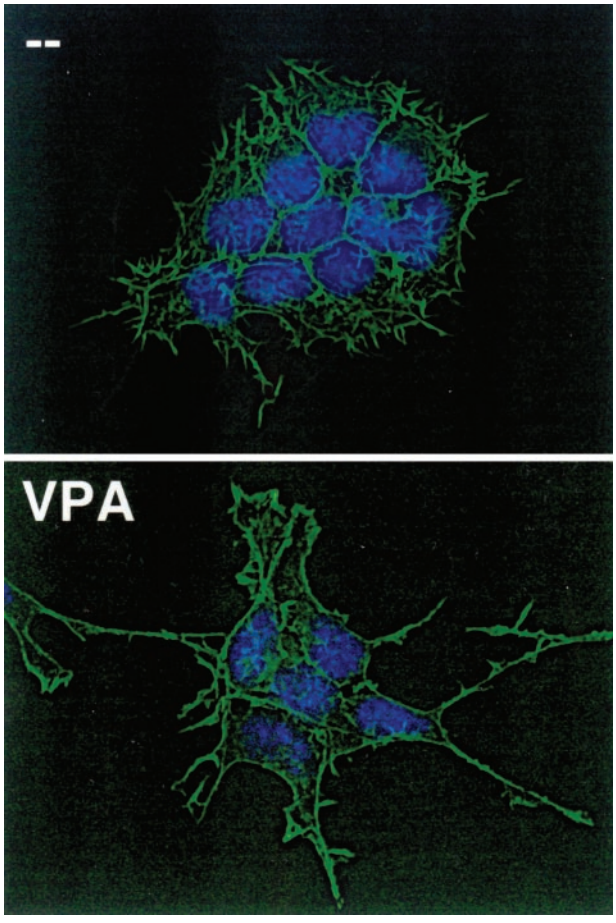

Fig. 1. VPA-induced changes in morphology of F9 teratocarcinoma cells. F9 cells were cultured on gelatin-coated plastic for 48 h in the presence or absence of 1 mM VPA. The F-actin cytoskeleton was stained with BODIPY-conjugated phallacidine (green fluorescence; Molecular Probes). Nuclei were stained with H33254. Culture dishes were cut to fit a microscope stage and micrographs of representative F9 cell colonies were taken using computer-aided deconvolution confocal imaging (Improvivon Labs, UK).

The VPA-induced type of F9 cell differentiation obviously was different from that induced by RA and did not fit the described marker gene patterns or morphology after differentiation by other agents. The search for a specific marker of VPA-induced F9 cell differentiation was guided by the neuronal-like morphology and the expression of a putative mediator of VPA effects [e.g., PPARδ (see below)] in neural crest cells (NCCs). NCCs originate from the neural folds and, after an extensive migration through the embryo, contribute to many organs, including the spinal ganglia and peripheral neural system, melanocytes, endocrine cells, and the facial skull (Erickson and Reedy, 1998; Groves and Bronner-Fraser, 1999, Mitchell et al., 1991). During embryogenesis, NCCs preferably express the AP-2α transcription factor and, thus, AP-2α may serve as a marker of NCC-like cell types. AP-2 protein was found not to be present in undifferentiated F9 cells using an antibody directed against AP-2α. However, it was highly induced by VPA. The time course (Fig. 3A) with the first detectable appearance of AP-2 protein after 1 day and a strong increase thereafter suggested that the induction of AP-2 by VPA required intermediate steps. To exclude cross-reactivity of the antibody with a nonrelated protein of the apparent molecular weight of AP-2, the inducibility of AP-2 protein was confirmed in an EMSA (Fig. 3B). The differentiation of F9 cells to AP-2 expressing cells is specific for VPA, before other differentiating compounds such as cAMP or the teratogen RA did not induce AP-2 expression, whereas butyrate only inefficiently did so (Fig. 3C). Also another teratocarcinoma cell line (i.e., P19) showed signs of VPA-induced differentiation. Morphological alterations were induced and proliferation was reduced even more efficiently compared with F9 cells (e.g., by 68% at 1 mM VPA and by 31% at 0.2 mM VPA).

Differentiation of F9 Cells by Teratogenic Rather Than Nonteratogenic VPA Derivatives. If F9 cell differ-

TABLE 1

Inhibition of F9 cell proliferation by VPA

Cells were counted in a hemocytometer and values are presented relative to the number at day 0. Values are mean ± S.D. from a total of six determinations in two independent experiments with triplicate cultures each. The ratio of VPA versus nontreated cultures is given. Significance of difference between control and VPA-treated cultures was tested by Student's *t* test. Thymidine incorporation (cpm) was determined by addition of 1 μCi of [^{3}H]thymidine per culture well for 12 h after 10,000 cells had been cultured for 1.5 days in the absence or presence of VPA on a microtiter culture dish. Values represent the relative recovery of insoluble radioactivity as mean ± S.D. from quadruple determinations. Similar results were obtained in three independent experiments.

	−VPA	+VPA (1 mM)	Ratio	p
Cell number				
Day 0	1	1	100%	
Day 1.5	5.8 ± 0.5	3.8 ± 0.5	66%	<0.001
Day 3	34.4 ± 2.8	15.6 ± 1.9	45%	<0.001
[^{3}H]Thymidine incorporation	1041 ± 8	538 ± 49	52%	<0.001

entiation reflects a process occurring during VPA-induced disturbance of embryonic development, the same type of differentiation should be induced by teratogenic derivatives of VPA, but not by nonteratogenic derivatives. A set of closely related derivatives, including the stereoisomers of 4-yn-VPA, was therefore tested for the induction of c-fos mRNA and AP-2 protein. The parental compound and teratogenic (*S*)-4-yn-VPA, but not the nonteratogenic or poorly teratogenic derivatives (*R*)-4-yn-VPA, 4-yn-4′-methyl-VPA, and 2-en-VPA, induced c-fos mRNA (Fig. 4A) and AP-2 protein (Fig. 4B). Moreover, proliferation was only inhibited by 1 mM (*S*)-4-yn-VPA, but not by (*R*)-4-yn-VPA [i.e., thymidine incorporation was reduced by 51 ± 3 and 8 ± 5%, respectively (data not shown)]. The identical stringent structural requirements for VPA derivatives to induce differentiation of F9 cells and disruption of embryonic development suggest that both effects are caused by the same primary action of VPA on cellular signaling molecules.

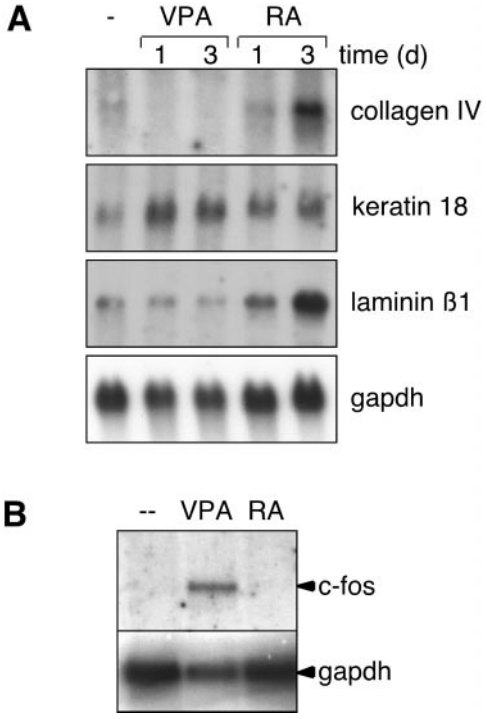

Fig. 2. VPA induces a specific type of F9 teratocarcinoma cell differentiation, which differs from that induced by retinoic acid. Differentiation of F9 teratocarcinoma cells was induced by exposure to 1 μM RA or 1 mM VPA for 1 or 3 days. A, abundance of the mRNAs for collagen (a1)IV, keratin 18, laminin β1, and gapdh for loading control was determined by Northern blot hybridization of 5 μg poly(A)$^+$ RNA using probes described previously (Auer et al., 1994). B, induction of c-fos mRNA by VPA (1 mM) or RA (1 μM) was tested after 18 h. The figures show one of three experiments with similar results.

TABLE 2

Quantitative evaluation of the differentiation marker gene expression

Northern hybridization signals were quantitatively evaluated by PhosphorImager analysis relative to the signal on the *gapdh* blot. Values of the experiment in Fig. 2 are shown and similar results were obtained in two additional experiments.

	Control	VPA		RA	
		1 d	3 d	1 d	3 d
Collagen IV	1	≤1	≤1	1.4	3.4
Keratin 18	1	2.9	3.2	1.7	1.7
Laminin β1	1	1.2	1.1	1.7	3.9

Activation of PPARδ by VPA and Teratogenic Derivatives. Prominent components in the cellular signaling network, which respond to small diffusible compounds, are the members of the steroid hormone receptor superfamily. Retinoid receptors are present in F9 cells (Boylan et al., 1995; Kastner et al., 1995) but are not likely to mediate the response to VPA because RA induced a different type of differentiation. PPARs are candidates of VPA-responsive receptors, because they respond to a wide variety of carboxylic acids (Göttlicher et al., 1992; Kliewer et al., 1997; Willson and Wahli, 1997). In a previous study, it was shown that even at a concentration of 0.5 mM, VPA activated a hybrid protein, comprising the DNA-binding domain of the glucocorticoid receptor and the ligand-binding domain of PPARδ (Göttlicher et al., 1992; Lampen et al., 1999). At a concentration of 4 mM, VPA activated PPARδ as efficiently as the established ligand iloprost (10-fold; data not shown). PPARδ was selectively activated by VPA and its teratogenic derivatives, but not by the nonteratogenic derivatives (Lampen et al., 1999). PPARα was activated only 3-fold even by 4 mM VPA, and PPARα activation was not sensitive to modification of the VPA-molecule [i.e., all derivatives used in this study activated PPARα to the same low degree (data not shown)]. The lack of acti-

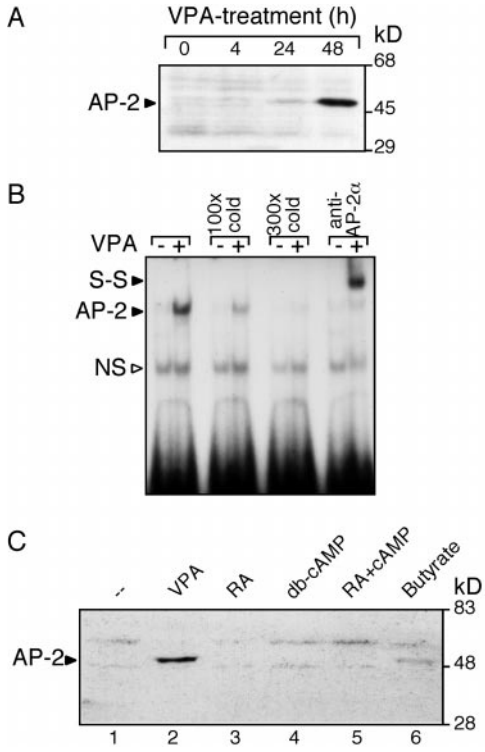

Fig. 3. Expression of the AP-2 transcription factor in VPA-differentiated F9 cells. A, time course of appearance of AP-2 protein during treatment with 1 mM VPA was followed by Western blot analysis of nuclear extracts. B, presence of AP-2 in nuclear extracts after 48 h of VPA treatment (1 mM) was confirmed in an EMSA by complex formation with an AP-2 binding DNA element. The specific complex (AP-2), a nonspecific protein-DNA complex (NS), and the mobility of the AP-2 complex in the presence of an antibody against AP-2 (supershift, S-S) are indicated. Specificity of binding was shown by preincubating nuclear extracts with a nonlabeled DNA element ("cold") before addition of the radioactive probe. C, appearance of AP-2 protein was tested in F9 cells differentiated for 40 h by various agents [e.g., VPA (1 mM), RA (1 μM), dibutyryl cyclic AMP (db-cAMP, 1 mM)], a combination of the latter, or sodium butyrate (1 mM). Coomassie staining of a part of the gel confirmed comparable loading of lanes. One representative of three similar experiments each is shown.

vation of the full-length glucocorticoid receptor served as negative control (data not shown). Fatty acids and other compounds are known to activate several forms of PPARs. In accordance with this promiscuity, PPARγ was activated selectively by VPA and the teratogenic derivatives, but not by nonteratogenic compounds. The same specificity was found as in the case of PPARδ (data not shown).

Based on the structure activity studies, both PPARγ and PPARδ may be qualified as mediators of F9 cell differentiation. Thus, it was crucial to determine the expression of PPAR forms in F9 cells. mRNA levels were determined by Northern blot analysis and compared with tissue RNA samples, because a previously published reverse transcription-polymerase chain reaction analysis did not allow firm quantitative interpretations. From among the three forms of PPARs, only PPARδ expression could be detected in F9 cells (Fig. 5A). The expression of functional PPARδ protein in F9 cells was supported by the DNA-binding activity to a PPARδ-specific DNA-binding site in F9 cell nuclear extracts (He et al., 1999) (Fig. 5B). The EMSA showed three complexes, two of which migrated closely together. The middle of these bands was considered to represent PPARδ based on the analysis of PPARδ antisense RNA expressing subclones of F9 cells (see below). Commercially available antibodies against PPARδ could not be used, because they did not recognize a preferential band of the expected size of PPARδ in Western blot analysis. As expected from the apparent lack of specificity and affinity, they did not induce a "supershift" in the EMSA with either of the complexes (data not shown). PPARδ-dependent gene expression in F9 cells and its inducibility by VPA were tested by transient transfection of a PPARδ-dependent reporter gene (Fig. 5C). The reporter gene was induced 4.9-fold in F9 cells by VPA. Overexpression of PPARδ and RXR enhanced basal reporter gene activities and inducibilty was slightly increased to 5.4-fold, suggesting that VPA activated the PPARδ-dependent gene expression, also if tested on the native full-length protein.

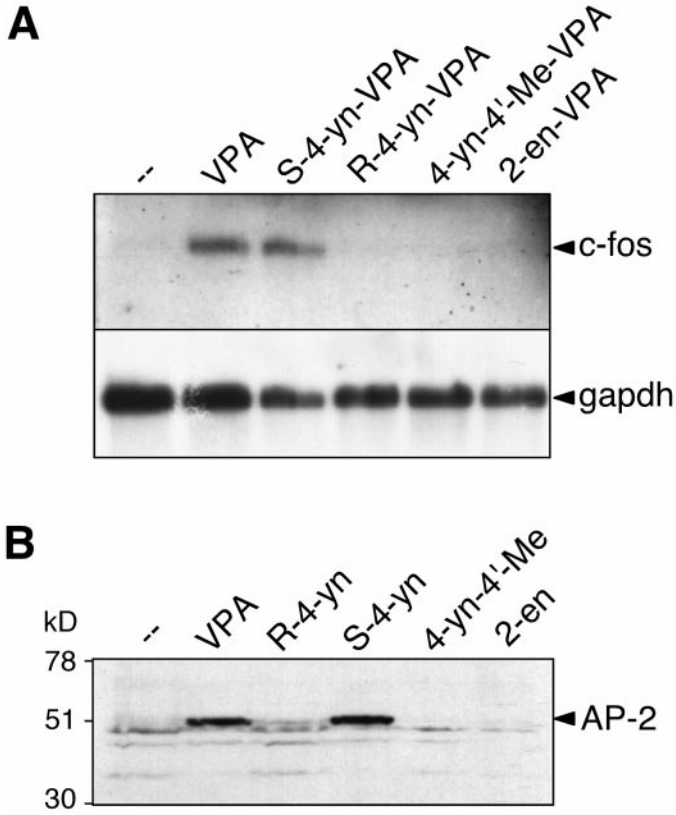

Fig. 4. Selective induction of c-fos mRNA and AP-2 protein by teratogenic, but not by nonteratogenic VPA derivatives. Inducibility of c-fos mRNA and AP-2 protein by derivatives of VPA was determined as described in Figs. 2 and 3. A, *c-fos* induction was determined after 8 h of exposure to 1 mM VPA or its derivatives and AP-2 protein was detected after 48 h (B). Similar results were obtained in three independent experiments and equal loading of samples was confirmed by hybridization with a *gapdh* probe or Coomassie staining of a part of the gel.

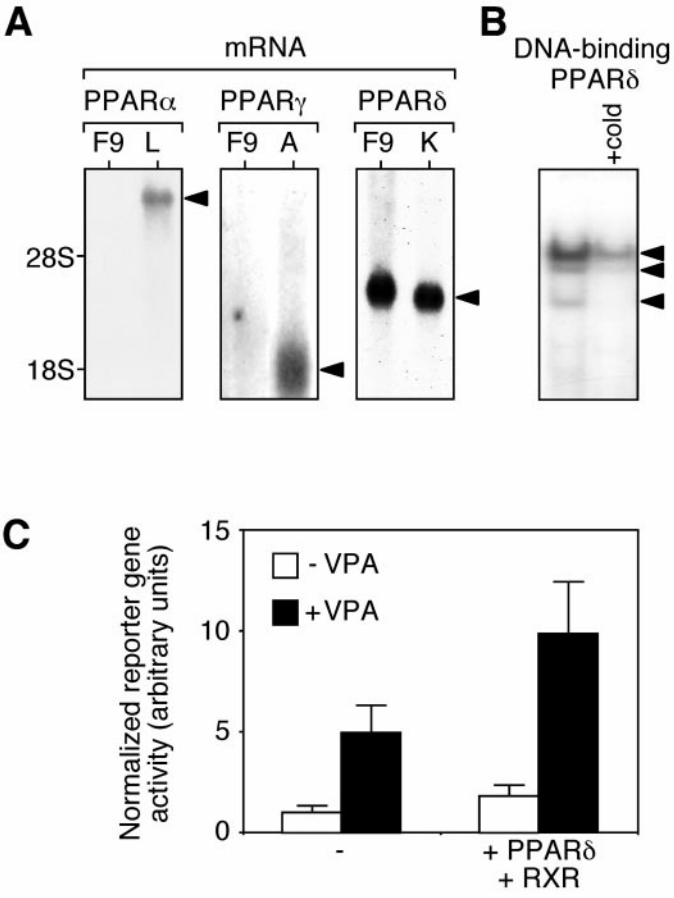

Fig. 5. Expression of *PPAR* isoforms in F9 teratocarcinoma cells. A, abundance of *PPAR*-isoforms was analyzed by Northern blot hybridization of 10 μg of poly(A)$^+$ RNA isolated from nontreated F9 cells. RNA isolated from mouse liver (L), adrenal (A, 14 μg total RNA), or kidney (K) served as control. The arrows indicate the sizes of the detected mRNA species (PPARα, 8.5 kb; PPARγ, 1.9 kb; PPARδ, 3.5 kb) calculated from the mobility of 18S and 28S rRNA. B, presence of PPARδ protein in nuclear extracts was tested by gel mobility shift analysis (right) using a DNA probe specifically selected for binding of PPARδ (He et al., 1999). Specific DNA binding was displaced by a 300-fold excess of unlabeled oligonucleotide (right). The lower half of the gel with the free probe is not shown. Similar results were obtained in two independent experiments. C, inducibility of PPARδ-dependent gene expression was tested by transient transfection of the PDRE4-Mluc reporter gene into F9 cells without or with coexpression of PPARδ and murine RXRα. Cells were left untreated (□) or treated with 1 mM VPA for 17 h (■) before analysis of reporter gene activity. Values are averages ± S.D. from a total of four determinations in three independent experiments that were normalized in comparison to noninduced reporter gene activities in the absence of receptor expression. Inducibility by VPA was significant ($P < 0.005$, Student's t test) in either pair of values. The effect of receptor coexpression was also moderately significant if untreated or VPA-treated samples were compared, respectively ($P < 0.05$).

Role of PPARδ in the Differentiation of F9 Cells. The activation of PPARδ suggests that this receptor might mediate VPA effects in F9 cells. PPARδ-deficient F9 cell clones were generated by the stable expression of antisense RNA to find out whether PPARδ caused the differentiation of F9 cells or whether PPARδ activation should rather be considered a surrogate marker for VPA action. Antisense RNA was expressed in a tetracyclin-dependent expression system (Tet-off) together with an expression cassette for luciferase. Three F9 cell clones of approximately 200 screened clones were identified, which expressed the transfected construct at high levels as assessed by luciferase measurements. However, none of the clones was responsive to tetracyclin. Considering the lack of suitable antibodies, the presence of PPARδ protein was assessed indirectly by EMSA with a PPARδ-specific probe (Fig. 6A). The EMSA pattern differed between wild-type F9 parental cells and cells ectopically expressing the tet-off *trans*-activator on one side and the three PPARδ antisense RNA expressing cells on the other side. One protein DNA complex (middle) was completely lost in the antisense cells, thus indicating that this band probably corresponded to a PPARδ-dependent complex and that antisense RNA expression was efficient in preventing PPARδ protein synthesis. These clones were resistant to the VPA-dependent decrease in the proliferation rate (determined as described in Table 1; data not shown) and to the induction of AP-2 protein (Fig. 6B). The result showed that PPARδ indeed serves as a limiting factor in F9 cell differentiation.

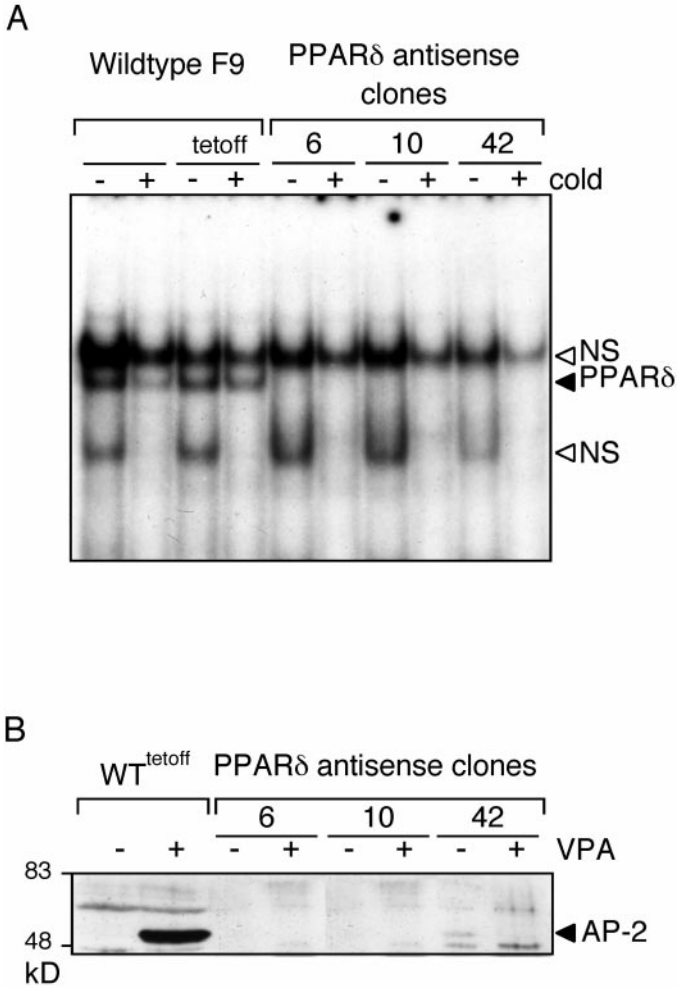

Fig. 6. PPARδ DNA-binding activity and induction of AP-2 by VPA in PPARδ antisense RNA expressing cells. PPARδ antisense RNA expressing F9 cells were tested for PPARδ-like DNA-binding activity in nuclear extracts and inducibility of AP-2 protein by VPA treatment. A, EMSA for DNA-binding of PPARδ was performed as described in Fig. 5. Wild-type cells and PPARδ expressing parental cells of the subclones ($F9^{tetoff}$) were analyzed in comparison to three subclones of $F9^{tetoff}$, which expressed an antisense RNA directed against PPARδ. Only the indicated band (◀) was considered to depend on PPARδ because the other bands (◁, N.S.) were not affected by antisense RNA expression. For reasons of resolution only the top third of the gel is shown. B, AP-2 was detected as described in Fig. 3 after treatment of cells for 48 h with 1 mM VPA. One representative of three independent experiments is shown.

Presence of PPARδ in the Developing Embryo. Studies in F9 cells suggest that inappropriate activation of PPARδ might also mediate VPA teratogenicity in vivo. This would require the expression of the receptor during the VPA-sensitive time of embryogenesis. Therefore, PPARδ expression was analyzed by RNA in situ hybridization. Eight days after conception, before the VPA-sensitive period, a weak expression only was found throughout the embryo cross-section (Fig. 7, A–E) with a slightly prominent signal in the neuroectoderm of the anterior neural fold (arrowheads in Fig. 7, A, B, D, and E). Although weak, the signal was clearly above the background obtained with a sense instead of the antisense probe (Fig. 7F). Using the antisense probe, a strong specific signal was found in extraembryonic tissues (Fig. 7, A and D). At 9.5 days after conception, ubiquitous staining was found throughout the whole embryo cross-section (Fig. 7, G–I) and at 10.5 days after conception, the expression seemed to be enhanced (arrowheads) along a line surrounding the neural tube as well as a more lateral line in the mesoderm on each side of the embryo (arrow, Fig. 7, K–M). This location reminds of the medial (along the neural tube) and lateral migration paths of NCCs. This finding, together with the VPA induction of the AP-2 transcription factor preferentially expressed in NCCs, suggested that the differentiation or function of NCCs could be the target of VPA action in the embryo.

Discussion

VPA Induces a Specific Type of F9 Cell Differentiation. The disruption of proper embryonic development by a small teratogenic chemical such as VPA during a precisely defined time window requires the compound to interfere with preexisting signaling pathways and the proper execution of cellular programs. This study was aimed at defining such a VPA-inducible cellular program by using the differentiation of pluripotent F9 embryocarcinoma cells as a model system. The main finding is that VPA induces a specific type of differentiation, which is characterized by a dramatic increase in the AP-2 transcription factor protein. Markers of other types of differentiation are missing. Parietal endoderm-like cells, for example, express *laminin β1* and *collagen IV* (Strickland et al., 1980; Kellermann et al., 1987; Sleigh, 1987; Edwards et al., 1988; Alonso et al., 1991). These markers are induced by RA, but not by VPA. The 3-fold induction of the keratin 18 mRNA by VPA could indicate induction of primitive or visceral endodem-like cells, which, however, are supposed to show a different morphology than that observed (Kellermann et al., 1987; Sleigh, 1987; Alonso et al., 1991). Neither does a protocol that induces neuronal differentiation [e.g., RA and dibutyryl cyclic AMP (Wartiovaara et al., 1984)

induce AP-2 expression (data not shown)]. Thus, VPA-induced differentiation to AP-2 expressing cells apparently defines a novel type of F9 cell differentiation. Because AP-2 expression is initiated during normal embryogenesis in premigratory NCCs at 8 days after conception (Mitchell et al., 1991; Schorle et al., 1996), F9 cells differentiated by VPA may resemble features of NCCs. The induction of AP-2 seems to involve cell type-specific elements, because RA induces AP-2 in NT2 tertatocarcinoma cells (Lüscher et al., 1989), but not in F9 cells treated with RA for up to 2 days.

Correlation of Induction of F9 Cell Differentiation with Teratogenicity. If the F9 cell model simulates events occurring during the disruption of proper embryonic development by VPA, structure-activity relationships for derivatives of VPA should be identical for both the differentiation of F9 cells and teratogenicity. Indeed, differentiation is only induced by the teratogenic, but not by the nonteratogenic compounds. This correlation also holds during a more extensive analysis of structure-activity relationships, including six teratogenic and six nonteratogenic derivatives of VPA, when testing an indirect marker of differentiation [e.g., the derepression of the Rous sarcoma virus promoter (Lampen et al., 1999)].

The induction of AP-2 in F9 cells by VPA gave rise to the question as to whether ectopic induction of this transcription factor may also cause a disruption of proper embryonic development in vivo. This seems possible before the sites affected by genetic deletion of AP2α (Schorle et al., 1996; Zhang et al., 1996) overlap to a major part with the sites of VPA-induced teratogenicity. Depletion or ectopic overexpression may disturb embryogenesis if one assumes that a regionally, timely, and quantitatively properly coordinated expression of AP-2 is required for normal embryonic development.

PPARδ in the Control of F9 Cell Differentiation. Specific induction of differentiation requires a target in the normal cellular signaling network, through which VPA acts on preexisting cellular programs by inappropriate activation or inhibition. The present data suggest that PPARδ is part of this VPA-sensitive signaling network. PPARδ mRNA is expressed in F9 cells, and functional protein is synthesized. PPARδ mRNA is also present in the embryo at the relevant time (Fig. 7). A comparable study in rat embryos (Braissant and Wahli, 1998) and an earlier analysis of RNA from whole mouse embryos (Kliewer et al., 1994) also support the sufficiently early presence of PPARδ in the embryo. Furthermore, VPA levels in vivo (Nau et al., 1981) reach those required for F9 cell differentiation (this study) and PPARδ activation in cell culture (Lampen et al., 1999), so that activation of the expressed receptor in the embryo is expected. Also PPARγ, but not PPARα, was activated in vitro selectively by VPA and the teratogenic derivative. These receptors may be relevant to other aspects of VPA action, such as peroxisomal proliferation, but they are not likely to mediate teratogenicity due to a lack of expression during the VPA-sensitive time window. Structure-activity relationships also indicate that activation of PPARδ and the described type of cell differentiation are not the mechanisms of the antiepileptic activity of VPA because some derivatives do neither induce differentiation nor activate PPARδ but nevertheless suppress epileptic seizures. To provide support for the proposed role of PPARδ in the differentiation of F9 cells, we generated three subclones that expressed PPARδ mRNA in antisense orientation. In these cells VPA did not induce any sign of differentiation. The most simple interpretation was that PPARδ mediates the effects of VPA. It could not be excluded, however, that depletion of PPARδ by antisense RNA expression primarily altered the state of F9 cell differentiation in a way that VPA sensitivity was lost indirectly. In either case, these findings suggest that PPARδ is part of the signaling network that controls F9 cell differentiation and directly or indirectly related to the action of VPA. Evidence for the proposed role of PPARδ in embryonic development and VPA teratogenicity still requires an analysis of PPARδ-deficient gene knockout mice.

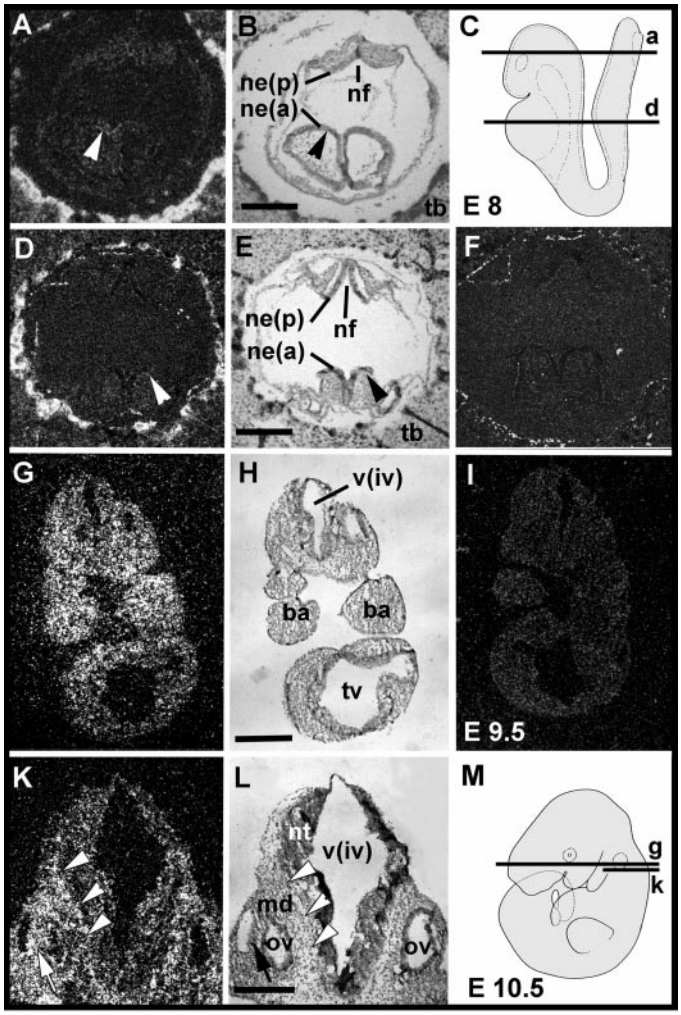

Fig. 7. In situ detection of PPARδ mRNA during the VPA-sensitive time window in embryonic development. PPARδ expression was detected by mRNA in situ hybridization in transversal sections of embryos at 8 (A–F), 9.5 (G–I), and 10.5 (K and L) days after conception. Left panels show autoradiographic developments of the antisense probe hybridization in dark field micrographs. Middle panels show the identical frames as bright field micrographs for orientation. Scale bars, 500 μm each. The right column either shows a schematic drawing for approximate orientation of the section planes (C and M), where the plane for the embryo 9.5 days after conception is also indicated in the corresponding region of the schematic drawing at 10.5 days after conception. F and I show hybridizations of serial sections to those shown in D and G. For control purposes they were hybridized with a sense RNA probe. Image processing for those frames exactly corresponded to that of the corresponding antisense RNA hybridized sections. Indicated structures are ba, branchial arch; md, mesoderm; ne(a), anterior neuroepithel (also arrow head); ne(p), posterior neuroepithel; nf, neural fold; nt, neural tube; ov, otic vesicle; tb, trophoblast; tv,.telencephalic vesicle; v(iv), forth ventricle.

Conclusions

To sum up, specific nuclear receptor activation and differentiation of F9 cells provide a model system for molecular and cellular events triggered by VPA and its teratogenic derivatives. Structure-activity relationships suggest that the effects described in F9 cells reflect at least some of the events that occur during VPA-induced disturbance of embryonic development in vivo. VPA teratogenicity is likely to involve complex cellular programs and the regulation of numerous gene activities. Nevertheless, the relatively simple model in F9 cells is suitable to define mechanisms of action and suggests that PPARδ plays a major part in the cellular response to VPA.

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