Volume 311, number 2, 102–106 FEBS 11630 October 1992

Retinoic acid-induced changes in differentiation-defective embryonal carcinoma RAC65 cells

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Received 25 May 1992; revised version received 24 August 1992

RAC65 is a mutant clone of mouse embryonal carcinoma cells, P19, which does not undergo terminal differentiation upon treatment with retinoic acid (RA). RAC65 cells express a truncated RA receptor α (RARα) which, however, does not fully explain their defect. Here we show that RAC65 cells exhibit an additional defect in RARα mRNA which may reflect a defect in RNA splicing. The parental and mutant cells also differ in their capacities to bind [^{3}H]RA into nuclear fractions and in expression of cellular RA binding protein (CRABP) mRNA after treatment with RA. The combined data suggest that the defect in RAC65 RARα results in reduced expression of the CRABP gene after RA treatment and, therefore, increased flow of RA into the nucleus.

Retinoic acid; Retinoic acid receptor; Embryonal carcinoma; Surface antigen; Differentiation

1. INTRODUCTION

Embryonal carcinoma (EC) cell lines provide a model system with which to analyze molecular mechanisms of early embryonic determination and differentiation [1]. The EC cell line, P19, can be induced to differentiate into neurons and glia when treated with retinoic acid (RA) [2]. RA functions through at least two distinct classes of nuclear receptors which belong to the steroid/thyroid hormone receptor family: RA receptors (RARs), including RARα, RARβ, and RARγ, and the retinoic X receptors (RXRs) [3–5].

The multiplicity of receptor subtypes underscores the need for a genetic approach to address their function. Recently, several groups have described that the P19 EC-derived RA-non-responsive mutant cells, RAC65, express a truncated RARα receptor [6–9]. Expression of the truncated RARα was, however, insufficient to fully confer RA non-responsiveness, suggesting that RAC65 cells carry another mutation(s) affecting RA-inducible genes [7]. To determine the additional defect(s) we set out to analyze biochemical changes induced in RAC65 cells by RA.

2. MATERIALS AND METHODS

2.1. *Cell cultures*

The origin and properties of the mouse EC cell lines (P19X1 and RAC65) have been described [10–13]. Cells were cultured in a 1:1 mixture of Eagles' minimal essential medium, supplemented with non-essential amino acids, 3 mM L-glutamine and 1 mM sodium pyruvate (H-MEMd), and RPMI-1640 medium. This medium was further supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), extra glucose (2.5 mg/ml), and 10% (v/v) heat-inactivated fetal calf serum. The cells were *Mycoplasma* free as determined by Hoechst staining [14]. To induce differentiation, the cells were harvested, washed and plated into culture medium supplemented with all-*trans* RA (Sigma).

2.2. *Immunofluorescence*

The cells were harvested and exposed to TEC-04 antibody [15] (ascites fluid diluted 1:100 in culture medium) for 30 min at room temperature, then washed and treated with swine anti-mouse IgG conjugated with fluorescein isothiocyanate (ÚSOL, Praha). The analysis was performed on a FACScan apparatus (Becton Dickinson).

2.3. *RNA blot analysis*

Total cellular RNA was isolated by the lithium chloride–urea procedure [16]. Poly(A)$^+$ RNA was selected by oligo-(dT) cellulose chromatography as described [17]. RNA was fractionated on 1% formaldehyde agarose gels and blotted onto nylon membranes (Hybond N; Amersham). The filters were hybridized with cDNA probes labeled with [α-^{32}P]dATP by the random priming method [17]. As probes we used a 2.1 kb *Eco*RI fragment of mRARα cDNA, a 1.95 kb *Bam*HI fragment of mRARβ cDNA, a 2.0 kb *Eco*RI fragment of RARγ cDNA [18], a 0.5 kb *Eco*RI–*Hin*dIII fragment of cellular RA binding protein (CRABP) cDNA [19], and a 1.6 kb *Pst*I fragment of rat α-tubulin cDNA [20]. Hybridization was performed at 42°C in 50% formamide using 'Church' buffer [21] supplemented with 0.5% low-fat milk, and salmon testes DNA (Sigma). The filters were washed in 2 × SSC, 0.1% SDS (4 × 10 min, room temperature) followed by 0.1 × SSC, 0.1% SDS (1 × 30 min at room temperature and 1 × 30 min at 65°C). After autoradiography, the blots were stripped in 0.1 × TE, pH 8.0, 0.05% SDS for 60 min at 80°C and sequentially re-hybridized with other cDNA probes.

2.4. *Retinoic acid uptake*

Cells (5 × 10^5) were placed in culture medium into 60 mm culture dishes and cultured for 24 h after which the medium was changed for medium with 1 μM RA or without RA. After another 24 h, the cells were washed and cultured for 3 h in serum-free medium supplemented

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Abbreviations: EC, embryonal carcinoma; RA, retinoic acid; RAR, retinoic acid receptor; CRABP, cellular retinoic acid binding protein; CAT, chloramphenicol acetyltransferase; RARE, retinoic acid responsive element.

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with 7.2 nM [11,12-^{3}H(N)]retinoic acid (2,060.9 GBq/mmol; NEN Research Products). Nuclear extract and cytosol were isolated as described [22]. Radioactivity of the extracts was measured in 5 ml of a scintillation liquid (SLD-31, Chemopetrol, Spolana Neratovice). Protein concentrations were assayed by the Coomassie blue protein assay [23].

2.5. *Transfection and chloramphenicol acetyl transferase (CAT) assay*

Transfection of plasmids into the cells was performed as described before [24]. 48 h after transfection the cells were tested in a CAT assay [17]. Efficiency of transfection was monitored by β-galactosidase activity [25].

3. RESULTS AND DISCUSSION

In a previous paper we have described that both P19X1 and RAC65 cells express on their surfaces SSEA-1/TEC-1 and TEC-4 antigens, and that their expression in P19X1 cells is down-regulated by RA [15]. When treated with RA, RAC65 cells exhibited neither changes in morphology nor a decrease in the expression of SSEA-1/TEC-1 ([12], our unpublished observations). TEC-4 is a more sensitive marker of EC cell differentiation than SSEA-1/TEC-1 [15] and, therefore, we analyzed TEC-4 expression in untreated and RA-treated RAC65 cells. Data presented in Fig. 1 show that RA induced a decrease in the expression of TEC-4 in RAC65 cells, however, this decrease was less extensive than that observed in P19X1 cells. This indicates partial responsiveness of RAC65 cells to RA; similar results were obtained with RAC65 subclones (not shown), excluding a role for RA-sensitive revertants in this differentiation.

RAC65 cells have been shown to carry a rearrangement affecting a gene encoding RARα [7]. Furthermore, it has been described that the level of expression of the normal RARα transcripts is dependent on the way RAC65 cells are cultured [8]. To determine whether RAC65 cells used in these experiments express an unusual RARα transcript, Northern blots of total RNA were hybridized with mouse RAR-specific cDNA probes. As shown in Fig. 2, RAC65 cells expressed two usual RARα transcripts of 3.0 and 4.0 kb, and a smaller transcript of 2.3 kb (RARα'). Surprisingly, the probe used also bound to a transcript of 9.1 kb. Neither the 2.3 nor 9.1 transcripts were present in RNA preparations from P19 EC cells. Unusual transcripts in RAC65 cells were also observed on Northern blots with poly(A)$^+$ RNA (Fig. 2, lanes c); the differences between

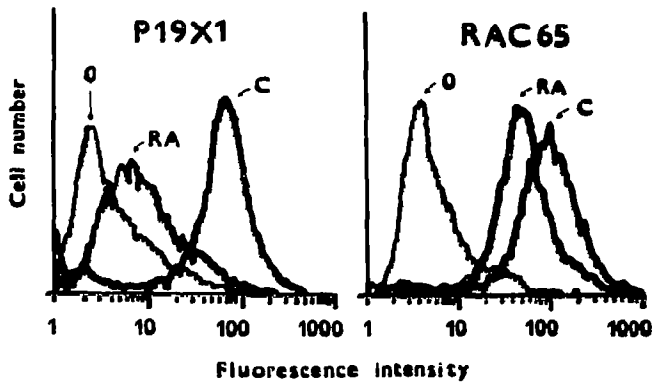

Fig. 1. Flow cytofluorimetric analysis of the expression of TEC-4 antigen in untreated (C) and RA-treated (RA, 0.5 μM, 3 days) P19X1 or RAC65 cells. The cells were labeled by indirect immunofluorescence using TEC-04 as a first layer antibody and fluoresceinated swine anti-mouse IgG as second layer antibody. A histogram obtained with RA-treated cells labeled with the second layer antibody only is also shown (0).

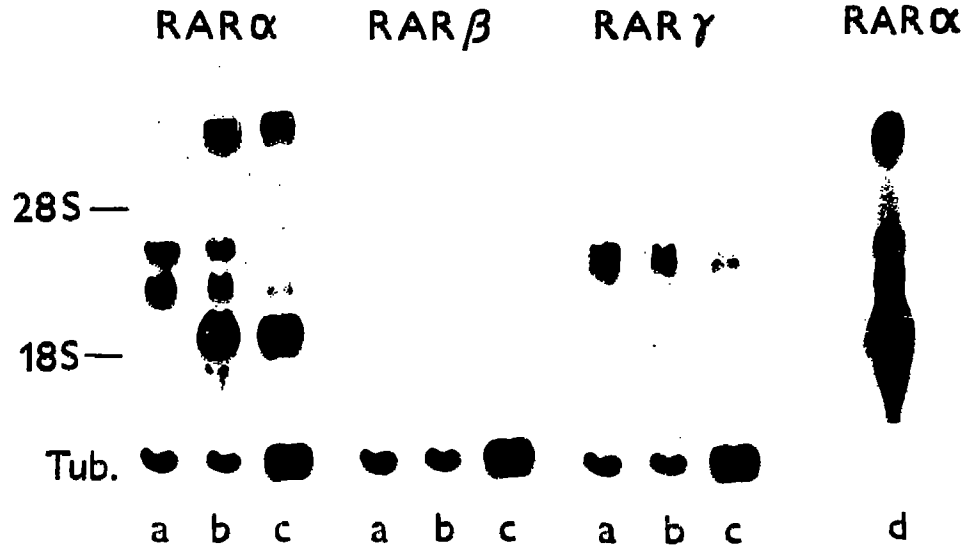

Fig. 2. Northern blot analysis of RARα, RARβ, and RARγ mRNA in P19X1 and RAC65 cells. (Lanes a) 20 μg total RNA isolated from P19X1 cells; (lanes b) 20 μg total RNA isolated from RAC65 cells; (lanes c) 4 μg poly(A)$^+$ RNA isolated from RAC65 cells. Membrane was sequentially probed with cDNA probes for mRARα, mRARβ, mRARγ and α-tubulin (Tub). (Lane d) 30 μg total RNA isolated from RAC65 cells cultured for 14 days in medium with RA. The position of 18 S and 28 S ribosomal RNA is indicated.

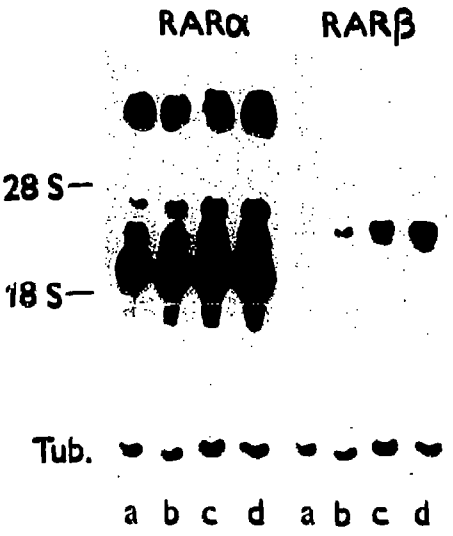

Fig. 3. Northern blot analysis of RARα and RARβ mRNA in RAC65 cells untreated (lane a) or treated for 24 h with RA at a concentration of 0.1 μM (lane b), 1 μM (lane c) or 10 μM (lane d). 20 μg total RNA was used per lane. Membrane was probed as described in Fig. 2.

Table 1
Incorporation of [^{3}H]RA into cytosol and nuclear extracts

Cells	Pretreatment with RA (1 μM)	[^{3}H]RA incorporation (dpm/μg)	
		Cytosol	Nuclear extract
P19X1	−	104 ± 3	220 ± 4
	+	110 ± 5	72 ± 8
RAC65	−	119 ± 5	319 ± 4
	+	122 ± 14	562 ± 31

Untreated (−) or RA-treated cells (+) were incubated with [^{3}H]RA and the uptake of radioactivity into cytosol and nuclear extracts were determined as described in section 2.4. The values are means ± S.E.M. from three experiments performed in triplicate.

total and poly(A)$^+$ RNA probably reflect different affinities of various RARα and RARγ transcripts to oligo-(dT) cellulose. The same unusual RARα transcripts (2.3 and 9.1 kb) were observed in RAC 65 cells which were subcultured for two weeks in the presence of 1 μM RA (Fig. 2d). The 9.1 kb RNA transcript in RAC65 cells, observed in all RNA isolates from RAC65 cells (7 independent isolates) and RAC65 subclones (4 isolates), was not described in previous studies [6–9]. Thus, RAC65 sublines cultured under our conditions differ from the sublines analyzed in other laboratories.

RAC65 cells, like P19 cells, expressed normal RARγ transcripts and did not express the RARβ transcript. Upon treatment of RAC65 cells with RA, the levels of all four RARα transcripts and RARβ transcript were enhanced (Fig. 3), supporting previous data on partial transcriptional induction of RARα and RARβ genes in these cells [6,7]. This induction is probably related to a

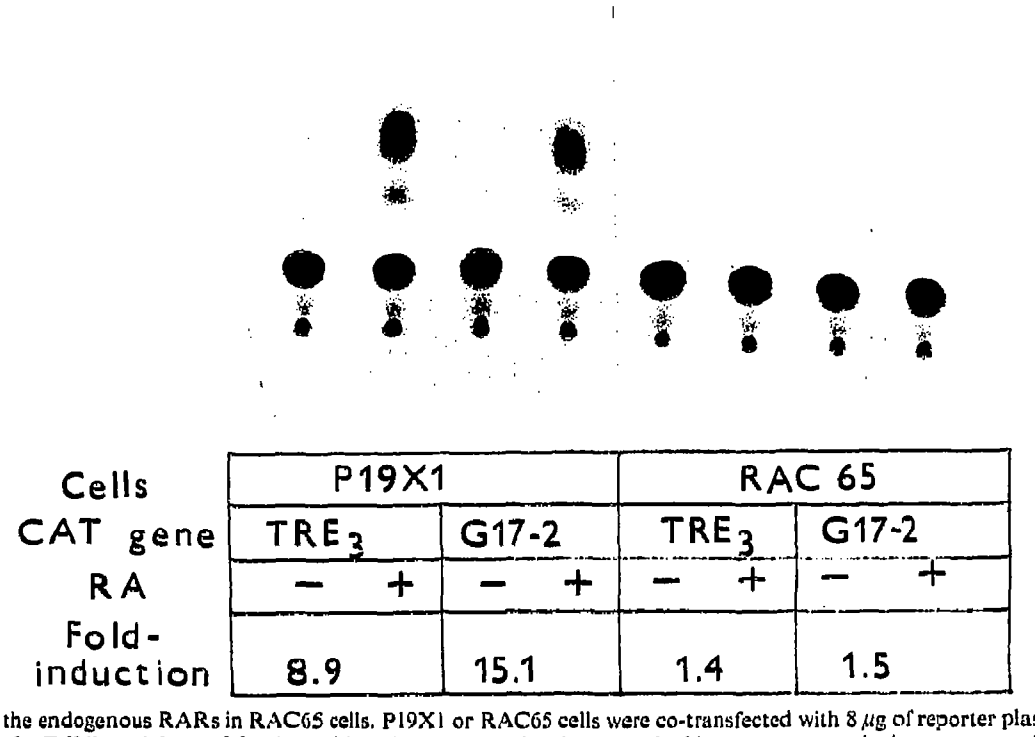

Fig. 4. Function of the endogenous RARs in RAC65 cells. P19X1 or RAC65 cells were co-transfected with 8 μg of reporter plasmids, $(TRE3)_3$-tk-CAT [18] or G17-2-CAT [26], and 2 μg of β-galactosidase *lacZ* gene under the control of human cytomegalovirus promoter [28]. The indicated samples were treated with RA (1 μM) and cell extracts were assayed for CAT and β-galactosidase activity (see section 2.5). Results are representative of a typical experiment of 8 performed.

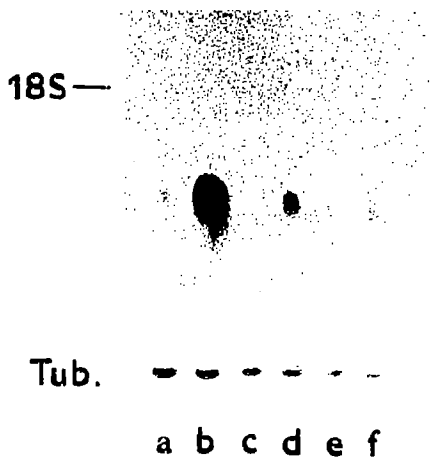

Fig. 5. Northern blot analysis of CRABP mRNA in P19X1 (lanes a,b), RAC65 (lanes c,d) or RAC65 subcultured for two weeks in 1 μM RA (lanes e,f). The cells were cultured in the absence (a,c,e) or in the presence of 1 μM RA for 40 h. 25 μg total RNA was loaded per slot. Membrane was hybridized with an *Eco*RI–*Hin*dIII fragment of CRABP cDNA and re-hybridized with the α-tubuline probe (Tub). The position of 18 S ribosomal RNA is indicated.

partial RA-induced decrease in the expression of TEC-4 antigen in these cells.

To verify that RA receptors in RAC65 cells are unable to activate transcription from synthetic RA-responsive elements (RAREs), we performed CAT assays using two different reporter plasmids: $(TRE3)_3$-tk-CAT [18] and G17-2-CAT [26]. The data presented in Fig. 4 indicate that transcription from both reporter genes transfected into P19X1 cells was dependent on the presence of RA (8.9- and 15.1-fold induction). However, although RAC65 cells were comparably transfectable with P19X1 cells, as determined by β-galactosidase assay, transcription from both reporter genes in RA-treated cells was significantly reduced (1.4- and 1.5-fold induction). Thus, RAC65 cells cultured under our conditions exhibit defects in their ability to initiate transcription from synthetic RAREs as a result of a defect in RARα. Similar results were obtained by other groups [7–9].

RAC65 cells express a mutant protein, RARα', that is 70 amino acids shorter than the wild-type receptor. These amino acids were lost from the C-terminal region that contains the RA-binding domain [7,9]. To determine whether RAC65 cells exhibit a defect in RA uptake, we compared incorporation of [^{3}H]RA into cytosol and nuclear extracts from untreated and RA-treated P19 and RAC65 cells and their subclones. Data presented in Table I indicate that the incorporation of [^{3}H]RA into cytosol was similar in both cell lines, and that pretreatment of the cells with RA had no effect on this parameter. Pretreatment of RA-responsive cells (P19X1) and RA-non-responsive cells (RAC65) induced, respectively, a decrease and an increase in [^{3}H]RA uptake into nuclear extracts.

The increased binding of [^{3}H]RA into nuclear extracts of RAC65 cells could result from an additional mutation in these cells, as predicted previously [7], or could reflect inability of RA-treated RAC65 cells to initiate transcription from the CRABP gene [19,27]. To distinguish between these two possibilities we analyzed CRABP mRNA in untreated and RA-treated P19X1 and RAC65 cells. Data presented in Fig. 5 show that RAC65 cells are defective in their ability to express the CRABP gene after treatment with RA. This defect is deeper in cells subcultured for two weeks in 1 μM RA (Fig. 5e,f).

The combined data suggest that the defect in RARα in RAC65 cells results in reduced expression of the CRABP gene after treatment with RA and, therefore, increased flow of RA into the nucleus.

Acknowledgements: We would like to thank Dr. P. Chambon for RAR cDNAs, Dr. L.N. Wei for sending us the CRABP cDNA fragment, Dr. J. Kvasnička for help with flow cytofluorimetric analysis, Ms. H. Klepalová for technical assistance and Dr. M. Rauscherová for help in preparing the manuscript. This paper was supported in part by a Czech Academy Grant, CS-55272.

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