Nuclear interactions of retinoic acid-binding protein in chemically induced mammary adenocarcinoma

Rajendra G. MEHTA, Wendy L. CERNY and Richard C. MOON

Laboratory of Pathophysiology, Life Sciences Division, IIT Research Institute, Chicago, IL 60616, U.S.A.

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Cellular retinoic acid-binding protein (CRABP) was detected in the nuclear fraction of *N*-methyl-*N*-nitrosourea-induced mammary cancers after the incubation of cytosol containing [³H]retinoic acid (RA)-bound CRABP with isolated nuclei. CRABP extracted from the nuclei in buffer containing 0.4 M-KCl sedimented as a 2 S component when subjected to sucrose-density-gradient analysis. [³H]RA-CRABP was found to be a prerequisite for the detection of nuclear binding, since the incubation of isolated nuclei or 0.4 M-KCl extract of the nuclei with [³H]RA did not result in any significant binding. Incubation of [³H]RA-CRABP at 25 or 30°C before incubation with the nuclei neither altered the sedimentation coefficient nor enhanced the nuclear binding compared with 0°C incubation. The tumour nuclei contained a saturable number of binding sites with a dissociation constant of 1.6×10^{-9} M. These results indicate that the action of retinoic acid in the target organ may be mediated by its interaction with the nuclei.

Dietary supplementation with certain retinoids significantly inhibits chemically induced mammary carcinogenesis in rats (Moon *et al.*, 1976, 1977, 1979; McCormick *et al.*, 1980, 1982). Although the mechanism of such inhibition by retinoids is unknown, it has been proposed that retinoid action may be mediated through its association with specific intracellular receptor proteins (Chytil & Ong, 1978, 1979), in a manner similar to that seen with steroid hormones (Jensen & DeSombre, 1973; Chan & O'Malley, 1976). In support of this hypothesis we have reported the presence of cellular retinoic acid-binding protein (CRABP) in normal and neoplastic mammary glands of the rat (Mehta *et al.*, 1980; Mehta & Moon, 1981), as well as human breast cancer (Mehta *et al.*, 1982a). Furthermore, it was also observed that a correlation exists between the ability of retinoids to suppress mammary carcinogenesis and the level of CRABP in the cytosol of mammary adenocarcinomas (Mehta *et al.*, 1982b).

Although extensive studies have been reported (Takase *et al.*, 1979) relating to the role of cellular retinol-binding protein in the interaction of retinol with the nuclear fraction, little is known about the interaction of retinoic acid (RA) with nuclear components. Although a CRABP that sediments as

a 2 S fraction on sucrose gradients has been reported for nuclei of retinoblastoma (Wiggert *et al.*, 1977), embryonal-carcinoma cells (Jetten & Jetten, 1979) and Lewis lung tumours (Sani & Donovan, 1979), a CRABP associated with the nuclear fraction of mammary tissues has not been studied. In the present paper we have attempted to characterize the interaction of retinoic acid with the nuclei obtained from *N*-methyl-*N*-nitrosourea-induced mammary tumours.

Experimental

Radioactive *all-trans*-retinoic acid (sp. radioactivity 2.8 Ci/mmol) was generously supplied by the Chemoprevention Section, Chemical and Physical Carcinogenesis Branch, National Cancer Institute, Bethesda, MD, U.S.A. Unlabelled *all-trans*-retinoic acid was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

Mammary tumours were induced by a single intravenous injection of 50 mg of *N*-methyl-*N*-nitrosourea per kg body weight to 50-day-old female Sprague-Dawley rats as described previously (Moon *et al.*, 1979; McCormick *et al.*, 1982). At necropsy the tumours were excised, a portion of each tumour was removed for histological processing, and the remainder was frozen in liquid N₂ and stored at -80°C in an ultrafreezer. Only

Abbreviations used: RA, retinoic acid; CRABP, cellular retinoic acid-binding proteins.

histologically confirmed adenocarcinomas were used for the study. Tumours were thawed, minced and homogenized gently in 50mM-Tris/HCl, pH 7.4. After centrifugation of the homogenate at 800g for 10min, the pellet was suspended in final concentrations of 1.25M-sucrose and 1.6mM-CaCl₂. This suspension was then filtered twice through three layers of cheesecloth and centrifuged at 50000g for 1h. The pellet so obtained was suspended in Tris buffer containing 0.23M-sucrose and 0.03mM-CaCl₂ and used for subsequent experiments; microscopic examination revealed little contamination with broken cells or connective tissue. The cytosol was prepared by centrifuging the 800g supernatant from the tumour homogenate at 105000g for 30min. The volume of the cytosol was adjusted so that the final concentration of protein in the cytosol was approx. 10mg/ml. CRABP in the cytosol was allowed to form a complex with [³H]RA by incubating 1μM-[³H]RA with 0.5ml of cytosol for 16h at 0–4°C in the dark. A parallel set of reactions included a 25-fold excess of unlabelled RA in addition to 1μM-[³H]RA and cytosol. The reaction mixture was then treated with a pellet of 1ml of dextran-coated charcoal and subjected to sucrose-density-gradient centrifugation. The gradients were centrifuged in a vertical tube rotor (TV 865) for 2h at 400000g. Radioactivity in each 10-drop fraction of the gradients was measured as described previously (Mehta *et al.*, 1980). Specific binding under the 2S region was calculated as pmol/mg of cytosol protein.

Studies involving nuclei were performed by incubating nuclei containing a known amount of DNA with cytosol containing a known concentration of [³H]RA–CRABP complex in a final volume of 1ml at 25°C for 30min. The nuclear pellet was subsequently separated by centrifuging the mixture at 800g for 10min; the pellet was resuspended with buffer, then centrifuged again at 800g for 10min. Retinoic acid-binding proteins were extracted from the nuclei with 2ml of Tris buffer, pH 7.4, containing 0.4M-KCl, whereas total [³H]-RA associated with nuclear fraction was extracted with 2ml of ethanol. Additional experiments were performed at several temperatures and reaction times to determine the optimal experimental conditions for interaction of retinoic acid with the nuclei; these are described below.

The concentration of DNA in the nuclei was measured by the diphenylamine procedure (Shatkin, 1969) and protein concentration in the cytosol was determined by u.v. spectrophotometry as described by Waddell (1956).

Results

The presence of CRABP in the cytosol of carcinogen-induced mammary tumours has been

previously reported (Mehta *et al.*, 1980; Trown *et al.*, 1980). Separation of cytosolic CRABP on the sucrose density gradients produces both a specific 2S binding protein as well as a non-specific 5S binding component as shown in Fig. 1(a). For obtaining CRABP from the nuclear fraction, nuclei were incubated with cytosol containing [³H]RA–CRABP at 25°C for 30min and subsequently were extracted with 50mM-Tris/HCl, pH 7.4, containing 0.4M-KCl. CRABP extracted from the nuclei separated as a specifically binding 2S component; no non-specific binding in the 5S region was observed (Fig. 1b). Under these conditions, approx. 6% (6.0 ± 1.2%, *n* = 9) of the total cytoplasmic CRABP was recovered in the KCl extract of the nuclei. In a separate experiment, nuclei prepared from fresh and frozen tumour tissue were compared for their properties to interact with retinoic acid. No difference was observed in the levels of CRABP between the nuclei prepared from either fresh or frozen tissues.

It has been reported that only about 20% of the [³H]steroid radioactivity associated with the nuclei is extractable with 0.4M-KCl after nuclear translocation of the steroid receptors, whereas nearly all the radioactive steroid can be extracted from the nuclei with ethanol (Lebeau *et al.*, 1974; Kalimi *et al.*, 1976). In steroid-exchange assays of nuclei, ethanol has routinely been used as extraction medium for quantification of nuclear steroid receptors (Anderson *et al.*, 1973; Clark *et al.*, 1980). We therefore compared the efficacy of 0.4M-KCl and ethanol for extracting retinoid receptor. It was observed that 0.4M-KCl extracted approx. 17% of [³H]retinoid radioactivity from the nuclei, whereas most of the radioactive retinoid was extractable with ethanol.

Optimal conditions for the nuclear interaction of retinoic acid were determined as described below. The time course of nuclear binding at two different temperatures was studied. Cytosol containing 40pmol of [³H]RA–CRABP, as determined by sucrose-density-gradient analysis, was incubated with 150μg of DNA equivalent of nuclei at 0 and 25°C for various times. Nuclei were extracted with ethanol and the radioactivity in the ethanol extract was measured. Results showed that maximum accumulation of CRABP in the nuclei occurred after 15min incubation at 25°C. Moreover, incubation of a constant amount of [³H]RA–CRABP with increasing amounts of nuclear suspension resulted in an increased accumulation of [³H]RA in nuclei until a concentration of 150μg of DNA per incubation; further increase in DNA concentration did not increase [³H]RA in the nuclei. In all the subsequent experiments a 30min incubation at 25°C with nuclei containing 100–200μg of DNA was used as optimal conditions.

To determine whether nuclei could bind free

Fig. 1. Sedimentation properties of [^3H]RA-binding proteins in the cytosol and nuclei of *N*-methyl-*N*-nitrosourea-induced mammary adenocarcinoma

Tumour cytosol was incubated with $1\ \mu\text{M}$ -[^3H]RA either alone (●) or in the presence of a 25-fold excess of unlabelled RA (○) for 16 h at 0°C in the dark. For CRABP in the nuclear fraction, nuclei equivalent to $250\ \mu\text{g}$ of DNA were incubated with pre-formed cytosolic [^3H]RA–CRABP for 30 min at 25°C . Nuclei were then washed and extracted in Tris buffer containing $0.4\ \text{M}$ -KCl as described in the Experimental section. After a brief treatment with dextran-coated charcoal, CRABP were layered on 5–20% (w/v)-sucrose gradients prepared either in Tris/EDTA buffer for cytosolic CRABP or in Tris/KCl buffer for nuclear CRABP. The gradients were centrifuged at $400\,000g$ for 2 h in a Sorvall TV 865 vertical tube rotor; fractions were taken from the bottom and the radioactivity in 10-drop fractions was measured. Haemoglobin (Hb) and myoglobin (Mb) were used as protein markers. The experiment was repeated over ten times.

[^3H]RA or whether pre-formation of cytosolic [^3H]RA–CRABP was essential for the detection of CRABP in the nuclei, the following experiment was carried out. Nuclei ($200\ \mu\text{g}$ of DNA) or $0.4\ \text{M}$ -KCl extract of nuclei were incubated with $1\ \mu\text{M}$ -[^3H]RA for 30 min at 25°C . Nuclei were then extracted with $0.4\ \text{M}$ -KCl-containing buffer and, after a brief treatment with a dextran-coated charcoal pellet, the nuclear extracts were subjected to sucrose-density-gradient analysis. No [^3H]RA radioactivity in the nuclear extracts was recovered in the 2S region. Under identical conditions, incubation of nuclei with cytosolic [^3H]RA–CRABP complex resulted in $0.4\ \text{M}$ -KCl-extractable CRABP from the nuclei which sedimented as a 2S component when subjected to sucrose-density-gradient centrifugation (Table 1). However, when nuclei were incubated with [^3H]RA–bovine serum albumin (which sediments as a 5S peak on the sucrose gradients) for 30 min at 25°C and subsequently extracted with $0.4\ \text{M}$ -KCl, it did not result in any peak upon sucrose-gradient centrifugation. These results would indicate that specific RA–CRABP complex was essential for the detection of CRABP in the nuclei.

In order to investigate the effects of temperature activation of [^3H]RA–CRABP complex on the nuclear binding, [^3H]RA was initially allowed to form a complex with cytosolic CRABP at 0°C . The cytosol was then divided into several aliquots and incubated at either 0 , 25 or 37°C for 30 min; after a brief treatment with dextran-coated charcoal, the reaction mixtures were centrifuged on sucrose gradients. No alteration in the sedimentation coefficient was detected. In a similar experiment, aliquots of cytosols containing [^3H]RA–CRABP complex were prewarmed at 25°C for 30 min and subsequently incubated with nuclei ($150\ \mu\text{g}$ of DNA); radioactivity in both KCl and ethanol extracts was measured. There was no enhancement observed in the nuclear binding as a result of prewarming the cytosol complex. Incubation of cytosol at 37°C for 30 min before incubation with nuclei resulted in decreased CRABP concentration (Table 1). These results indicated that temperature activation of RA–CRABP may not be essential for the nuclear interaction of retinoic acid.

To evaluate the nature of retinoic acid binding to the nuclear fraction, tumours which did not contain

Table 1. Nuclear uptake of [^3H]retinoic acid under various experimental conditions

Expt. no.	Reaction	Time and temperature of activation	Time and temperature of incubation	[CRABP]* (pmol/100 μg of DNA)
			for nuclear binding	
1	[^3H]RA + buffer + nuclei	—	0°C, 30 min	<1
			25°C, 30 min	<1
	[^3H]RA + 0.4 M-KCl + extract of nuclei	—	0°C, 30 min	<1
			25°C, 30 min	<1
	[^3H]RA-CRABP†	0°C, 30 min	25°C, 30 min	7.49
		25°C, 30 min	25°C, 30 min	7.68
		30°C, 30 min	25°C, 30 min	6.89
2	[^3H]RA-bovine serum albumin	—	25°C, 30 min	<1
			25°C, 30 min	2.45
			25°C, 30 min	2.49
			25°C, 30 min	2.00
			25°C, 30 min	0.85

* Concentrations of CRABP in the nuclei were determined by using sucrose-density-gradient analysis.

† [^3H]RA-CRABP complex was prewarmed at the temperatures indicated before incubation with nuclei at 25°C for 30 min.

Fig. 2. Titration of [^3H]RA binding sites in the nuclear fraction

(a) Sucrose-density-gradient profile of carcinogen-induced mammary tumour; cytosol was incubated with [^3H]RA either alone (●) or in presence of a 25-fold excess of unlabelled RA (○). Note that no 5S component was present. (b) Increasing concentrations of [^3H]RA-CRABP were incubated with constant amounts of nuclei, either alone or in presence of a 10-fold excess of unlabelled RA-CRABP. Radioactivity in an ethanol extract of nuclei was measured. Total (○), non-specific (Δ) and specific (□) binding of [^3H]RA to the nuclear fraction is presented. (c) The Scatchard analysis of the data shown in (b). The experiment was repeated three times.

any non-specific binding in the 5S region were selected (Fig. 2a). Nuclei (100 μg of DNA) were incubated with increasing concentration of [^3H]RA-CRABP in the cytosol for 30 min at 25°C

either alone, or in presence of approx. 10-fold excess unlabelled RA-CRABP. Since it is not possible to quantify unlabelled RA-CRABP accurately under the present experimental conditions, only an

approximation can be made. The nuclei were washed with buffer twice and extracted with ethanol. Radioactivity in the ethanol extract was measured. The difference between total and non-specific ethanol-extractable radioactivity was considered as specific binding (Fig. 2b). Saturation of nuclear sites occurred at a concentration of about 30 nM-cytosolic [^3H]RA-CRABP complex. By using Scatchard analysis of the data, a dissociation constant (K_d) of 1.68×10^{-9} M and the number of binding sites (4.6 pmol per 100 μg of DNA) can be estimated. The correlation coefficient of the plot was calculated to be 0.98. These results indicate that RA-CRABP recovered from the nuclei represent a single class of binding sites.

Discussion

In the present study, CRABP from both cytosol and nuclei sedimented as 2S components on the sucrose density gradients; however, non-specifically associating 5S component was not observed in the profile of the nuclear CRABP. Similar results have been reported by other investigators (Sani, 1977; Wiggert *et al.*, 1977; Sani & Donovan, 1979). Moreover, [^3H]RA-CRABP was found to be a prerequisite for the detection of nuclear binding components; incubation of [^3H]RA with either isolated nuclei or nuclear extract did not result in any significant binding. A similar requirement of cytosolic RA-CRABP complex for nuclear interaction has been reported in embryonal-carcinoma cells (Jetten & Jetten, 1979) as well as for retinoblastoma cells (Wiggert *et al.*, 1977).

It has been established that the cytoplasmic oestrogen receptors prepared from immature rodent uteri undergo temperature activation before translocating into the nuclei (Jensen & DeSombre, 1973). Similar temperature activation has also been reported for the oestrogen receptors in the mammary gland of lactating rats (Park & Wittliff, 1977). The sedimentation coefficient of oestrogen receptors in high-salt buffers is altered from 4S to 5S in uterine cytosol (Jensen & DeSombre, 1973), whereas no difference was observed in sedimentation for the mammary cytosol (Park & Wittliff, 1977). In both uteri and mammary glands, oestrogen binding to the nuclear components was significantly increased after the activation. Experiments were therefore conducted to determine if temperature activation of CRABP was required for the nuclear interaction, and it was observed that, unlike the case of oestrogen receptors, no enhancement in the nuclear binding was evident as a result of prewarming the cytosol containing RA-CRABP complex.

It had been reported previously that retinol-bound cellular retinol-binding protein can interact with rat liver nuclei in a specific manner, resulting in

saturable retinol binding with nuclei (Takase *et al.*, 1979). In the present study, saturation of RA binding in mammary-tumour nuclei with increasing amount of cytosolic RA-CRABP was observed. Scatchard analysis of the results indicated that the association of RA-CRABP in the nuclei is of high affinity, with a dissociation constant of 1.6×10^{-9} M.

Although the significance of CRABP in the interaction of retinoic acid with the nuclear fraction is unknown, it is evident from the present report that pre-formation in the cytosol of a complex CRABP with RA is essential for its interaction with the nuclear compartment. It does not, however, explain whether the cytosolic RA-CRABP complex enters the nucleus or whether its formation in the cytosol is essential to deliver RA to the nucleus. Nevertheless, these results support the thesis that the action of RA may be receptor-mediated in a fashion similar to that known for steroid hormones.

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