Cellular uptake of retinoic acid *in vitro*

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Incubation of chick embryo skin and mouse colon tumour 26 with [^3H]retinoic acid resulted in the formation of a complex of retinoic acid and its cellular binding protein both in cytosol and in nuclei. Formation of the ligand–protein complex was temperature-dependent and increased with increases in retinoic acid concentration in the incubation medium. About 3–8% of the ligand present in the cytosol was associated with the nuclei.

Retinoic acid and its synthetic analogues have been used successfully in decreasing the incidence or inhibiting the growth of chemically-induced tumours in laboratory experiments (Sporn *et al.*, 1977) and in clinical trials (Evvard & Bollag, 1972). Because of these anti-tumour activities, retinoic acid and its derivatives have recently gained increased attention. A retinoic acid-binding protein has been detected in the cytosols of several retinoid-responsive tissues (Sani & Corbett, 1977). This protein from chick-embryo skin cytosol has been purified to apparent homogeneity (Sani & Banerjee, 1978). A similar binding protein has also been detected in the nuclei of chick-embryo skin (Sani, 1977), retinoblastoma cells (Wiggert *et al.*, 1977) and embryonal carcinoma cells (Jetten & Jetten, 1979). Like the retinoic acid-binding protein from cytosol, the nuclear binding protein from chick-embryo skin also shows ligand specificity and has a sedimentation coefficient of 2S (Sani, 1977).

Available evidence suggests that the biological activity of retinoic acid is mediated by its binding protein (Sani *et al.*, 1978). Thus the first step in the action of retinoic acid is the formation of a complex between retinoic acid and its binding protein. In the present study we have measured the formation of such a complex in the cytosol of chick-embryo skin under different conditions of incubation of the tissue with [^3H]retinoic acid. Data pertaining to the formation of a nuclear binding protein–ligand complex are also presented.

Materials and methods

Tissues

Skin was collected from 14-day chick embryos. Colon tumour 26 was produced by subcutaneous implantation of trocar fragments of the tumour into mice as described elsewhere (Sani & Titus, 1977).

The tumours that developed at the primary site were removed 16–21 days after the implantation. All tissues were repeatedly washed in 0.03M-sodium phosphate buffer, pH 7.2, containing 100mM-NaCl to remove as much as possible the contaminating albumin, which also binds retinoic acid and may hence interfere with the quantification of the specific binding protein.

Incubation

The tissues were minced into small pieces and suspended in Eagle's modified medium without serum (pH 7.0) (Sani & Titus, 1977). The suspensions were pre-equilibrated at the incubation temperature and appropriate amounts of [^3H]retinoic acid were added. The incubations were carried out in the dark with frequent stirring. Incubations were terminated by chilling the suspensions rapidly and washing the tissues twice with 50mM-Tris-HCl (pH 7.5)/25mM-KCl/2mM-MgCl₂/0.5M-sucrose (buffer N); the tissues were collected by centrifugation at 10000g for 10 min.

Tissue fractionations

The tissues were suspended in 20 vol. (w/v) of buffer N. The suspensions were homogenized by 5–7 strokes in a VirTis Teflon homogenizer with intermittent cooling. The homogenates were filtered through three layers of cheesecloth and centrifuged at 800g for 10 min. The supernatant was further clarified by centrifugation at 100000g for 1 h. The cytosol preparations were stored at –20°C until used. For the preparation of the nuclei, the 800g pellet was resuspended in 20 vol. of buffer N as described above but containing 1.75M-sucrose and was homogenized by several strokes of a Teflon homogenizer. The homogenate was centrifuged at 25000g for 30 min to pellet the nuclei, which were found by microscopic examination to be purified to

homogeneity. For further purification of the nuclei, the pellet was resuspended in buffer N containing 1.25 M-sucrose, layered on the top of buffer N containing 1.75 M-sucrose and 1% Triton X-100, and was centrifuged at 25 000 g for 1 h.

Detection of retinoic acid-binding protein

To determine the extent of ligand binding to retinoic acid-binding protein, the cytosol preparations were dialysed overnight against 0.03 M-Tris/HCl, pH 7.2, containing 0.5 mM-dithiothreitol to remove free retinoic acid and 0.3 ml samples containing 3 mg of protein were centrifuged on linear 5–20% (w/v) sucrose gradients for 18 h at 180 000 g. The gradients were fractionated and counted for radioactivity in a liquid-scintillation counter. The retinoic acid-binding protein peaks corresponding to sedimentation coefficients of 2S were located from the radioactivity profiles of the gradient by using bovine serum albumin and ovalbumin as external standards (Sani & Hill, 1976). For the measurement of nuclear retinoic acid-binding protein, the purified nuclear preparations were incubated with 10 mM-Tris/HCl (pH 7.5)/0.3 M-KCl/1 mM-EDTA for 1 h at 20°C and were then homogenized with a Teflon homogenizer. The nuclear extract after centrifugation at 100 000 g for 1 h was analysed for the presence of the [³H]retinoic acid-binding protein complex by sucrose-gradient sedimentation as described above. Protein concentrations were determined as described by Lowry *et al.* (1951).

all-trans-[11,12-³H]Retinoic acid (1.11 Ci/mmol) supplied by Hoffman-La Roche, Nutley, NJ, U.S.A., was further purified by t.l.c. on silica gel G plates with benzene/chloroform/methanol (4:1:1 by vol.) as solvent and authentic retinoic acid as standard. Purified retinoic acid was stored at –60°C and was used within 2 weeks of purification. Other chemicals were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

Results and discussion

It is widely accepted that the most important action of retinoids is on cellular differentiation and that the site of action is probably the nuclei of the target cells. It is reasonable to presume that the process is mediated by a protein that specifically binds retinoic acid (Sani & Hill, 1976). A protein with a high affinity for retinoic acid was found by Sani & Donovan (1979) to be located mainly in the cytoplasm and nuclei. An early cellular event in retinoic acid action is the entry of the circulating retinoid into the cytoplasm and formation of a complex with the binding protein. To test the effect of temperature on the formation of such a complex, chick-embryo skins, 5 g each, were incubated in 20 ml of Eagle's modified medium containing 1 μ M-[³H]retinoic acid for 2 h at 0, 20 or

37°C. The incubations were terminated by quick chilling and immediate centrifugation followed by washing, since preliminary experiments indicated that incorporation of the ligand was minimal at 0°C. Fig. 1 illustrates the effect of the temperature of incubation on the formation of the binding protein-[³H]retinoic acid complex in the cytosol of chick-embryo skin. We have previously shown that, in the absence of albumin, the size of the radioactivity peak at 2S is a reliable index for the determination of the retinoic acid-binding protein concentration in an extract (Sani *et al.*, 1978). On the basis of this criterion, the formation of the binding protein–ligand complex at 37°C and at 20°C is 10-fold and 3-fold, respectively, higher than at 0°C. A similar experiment with colon tumour 26 also exhibited temperature dependency, but to a lesser extent. At 37°C the incorporation of the ligand into the binding protein of the cytosol was only about 3-fold that at 0°C. Whether such a temperature dependency exists in the formation of the nuclear protein–retinoic acid complex is not presently known.

The time dependence of the incorporation of [³H]retinoic acid by the cytosol binding protein of chick-embryo skin was also studied. As indicated by the size of the radioactivity peak at 2S, retinoic acid

Fig. 1. Sucrose-density-gradient-centrifugation patterns of cytosol (3 mg of protein) prepared from chick-embryo skin after incubation for 2 h in Eagle's modified medium containing 1 μ M-[³H]retinoic acid at different temperatures.

●, 0°C; ○, 20°C; △, 37°C.

Table 1. *Distribution of [³H]retinoic acid in the cytosol and nuclei of chick-embryo skin*

Chick-embryo skins (5g each) were incubated at 37°C in 20ml of Eagle's modified medium containing different concentrations of [³H]retinoic acid (1.11Ci/mmol). After 2h cytosol and nuclei were prepared from the tissues. Radioactivity in the cytosol was counted directly. [³H]Retinoic acid in the nuclei was extracted with absolute ethanol and counted for radioactivity.

Concentration of [³ H]retinoic acid in incubation medium (M)	10 ⁻⁵ × [³ H]Retinoic acid binding to cytosol (d.p.m.)	10 ⁻³ × Total nuclear uptake (d.p.m.)	Ratio of nuclear/cytosolic binding capacities (%)
4 × 10 ⁻⁸	2.0	15.0	7.5
1 × 10 ⁻⁷	1.5	4.0	2.6
2 × 10 ⁻⁷	2.2	10.0	4.5
1 × 10 ⁻⁶	16.0	43.0	2.7
2 × 10 ⁻⁶	30.0	163.0	5.4
4 × 10 ⁻⁶	60.0	234.0	3.9

Fig. 2. *Sedimentation patterns of cytosol (3 mg of protein) prepared from chick-embryo skin after incubation for different periods at 37°C in 20 ml of Eagle's modified medium containing 1 μM-[³H]retinoic acid*
○, 30 min; ▲, 60 min; △, 90 min; ■, 120 min; □, 240 min.

incorporation increased almost linearly up to 2h (Fig. 2). When incubation is extended further, there is a considerable decrease in the size of the 2S peak. Small peaks at 5S, which appeared in the profiles of some of the extracts, were due to the presence of contaminating serum albumin. Whether the decline in retinoic acid uptake by the cytosol at 4h is due to its metabolism during incubation or due to translocation of the ligand or the ligand-protein complex to the nuclei is not known.

The effect of increasing concentrations of retinoic acid on the formation of ligand-protein complex and the distribution of retinoic acid in the cytosol and in the nuclei was studied in a separate experiment (Table 1). Except at very low concentrations, the size of the peak at 2S increased linearly with the increase in ligand concentration in the medium (results not shown). The cytosolic and nuclear uptake also increased correspondingly. To test the saturation of the available sites on the binding protein, 300pmol of [³H]retinoic acid was further added to the cytosol prepared from chick-embryo skin preincubated with 1 μM-[³H]retinoic acid for 2h at 37°C. The mixture was incubated for 1h at 0°C, dialysed and subjected to sucrose-density-gradient centrifugation. The size of the peak at 2S in the resulting radioactivity profile increased about 2-fold (results not shown). Apparently, even after 2h incubation at 37°C with 1 μM-[³H]retinoic acid only about half of the retinoic acid-binding sites were occupied. We have reported earlier that half of the binding sites of cytoplasmic retinoic acid-binding protein from chick embryo skin bind *p*-chloromercuribenzenesulphonic acid loosely, so much so that the complex can be dissociated by dialysis; the other half of the binding sites bind the mercurial compound tightly (Sani & Banerjee, 1978). It would be of interest to see if there is any correlation between these two events. The presence of detectable amounts of retinoic acid in the nuclei under subsaturating ligand concentrations in the cytosol shows that total saturation of the binding sites of cytosolic retinoic acid-binding protein is not required for nuclear uptake. Under the conditions described about 3–8% of the retinoic acid bound in the cytosol appeared in the nuclei. It would be of interest to compare these values with the pattern of subcellular distribution *in vivo*.

The nuclear extract obtained after incubation of chick-embryo skin at 37°C for 2h in Eagle's modified medium containing 1 μM-[³H]retinoic acid

Fig. 3. Sedimentation patterns of nuclear extract (1 mg of protein) from chick-embryo skin after incubation of the tissue at 37°C for 2 h in the presence of 1 μ M-[³H]retinoic acid

●, Without dialysis; ○, after 22 h dialysis against 0.03 M-Tris/HCl, pH 7.2, containing 0.5 mM-dithiothreitol.

was analysed for the binding protein with and without dialysis. The nuclear extract produced a radioactive peak at 2S, but the binding of the ligand was loose and the bound retinoic acid dissociated from the complex on dialysis as shown by the disappearance of the radioactivity peak at 2S (Fig. 3). In contrast, at least part of the retinoic acid bound to the protein derived from the cytosol is stable to dialysis.

The results presented here add evidence to the hypothesis proposed earlier from this laboratory about the similarity of the modes of action of retinoic acid and of steroid hormones (Sani & Hill, 1976). The first step in the action of retinoic acid is the entry of the ligand into the cytoplasm and formation of a complex with the binding protein. In the tissues tested, this is a temperature-dependent event. The second step of the process may be the association of the ligand or the ligand-protein complex with the nuclei. What regulates this phenomenon is not known.

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