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PREFERENTIAL SOLUBILIZATION OF TRITON X-100 RESISTANT NUCLEAR GLUCOCORTICOID RECEPTORS BY DEOXYRIBONUCLEASE I

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SUMMARY: Nuclei, prepared by hypo-osmotic lysis from rat thymocytes that had been incubated at 37°C with 20 nM [1,2,4-^{3}H] triamcinolone acetonide, contained 2600-2800 stably and specifically bound steroid molecules per nucleus, 70-80% of which were retained after treatment with Triton X-100. DNAase I was more effective than DNAase II in solubilizing detergent-resistant glucocorticoid-receptor complexes from nuclei at both 0°C and 37°C; at 0°C, DNAase I digestion released 50-55% of the bound steroid with only 10% digestion of DNA. These results imply that the glucocorticoid-receptor complex is preferentially associated with transcriptionally active chromatin.

Glucocorticoids, in common with other steroid hormones, appear to regulate gene expression by means of an initial interaction with a steroid-specific cytoplasmic receptor that subsequently undergoes transformation and translocation to the cell nucleus, where association with the genome results in changes in the activity of one or many genes (1-3). Although a considerable body of evidence implicates DNA in the nuclear binding of glucocorticoid-receptor complexes (3-6), this binding appears to be heterogeneous (7-9) and may involve several moieties and categories of binding sites. Nuclease digestion techniques have been used by a number of investigators to study the nuclear localization of glucocorticoid receptors, but conflicting results have been reported (4,7,9-14). In the work reported here, using rat thymocytes, we have identified two stable classes of nuclear-associated glucocorticoid

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receptors by their sensitivity to detergent extraction, and have used digestion with DNAases I and II to study the localization of the detergent-resistant class of nuclear-bound receptors.

MATERIALS AND METHODS

Isolation of cells and nuclei: Thymus tissue was obtained from 8-12 week-old male Sprague-Dawley rats that had been adrenalectomized 1-2 weeks prior to use. Thymocyte suspensions (10^8 cells/ml) were prepared in tissue culture medium 199 as described previously (15). Nuclei were obtained by hypo-osmotic lysis of thymocytes, by dilution into 25 vol. of 2 mM $MgCl_2$ at 0°C for a period of time determined by experimental design. Purified nuclei were prepared by homogenization of crude nuclear preparations, obtained after hypo-osmotic lysis for 60 min at 0°C, in STKM buffer (0.25 M sucrose, 50 mM Tris HCl, 25 mM KCl, 5 mM $MgCl_2$, pH 7.4) containing 0.1% (v/v) Triton X-100 at 0-4°C, followed by two washes in 20 vol. of STKM buffer.

Measurement of nuclear steroid binding: Thymocytes were incubated with 20 nM [1,2,4-^{3}H] triamcinolone acetonide (20 Ci/mmol; Amersham International, Amersham, Bucks., U.K.), in the presence and absence of 2 μM non-radiolabelled dexamethasone, for 1 h at 37°C. Nuclei were prepared and collected on Whatman GF/C glass fibre filters under vacuum, and washed with 5 x 5 ml of 2 mM $MgCl_2$ at 0°C. Radioactivity retained on the filters was determined by liquid scintillation counting of the filters in 10 ml of emulsifying scintillant 299 (Packard Instrument Co. Caversham, Berks., U.K.), and specific glucocorticoid binding was calculated as the difference in binding between parallel samples incubated in the presence and in the absence of 2 μM dexamethasone.

Nuclease digestion procedures: Purified nuclei were prepared from cells that had been incubated with 20 nM [1,2,4-^{3}H] triamcinolone acetonide in the presence and absence of 2 μM dexamethasone. Nuclei were incubated in STKM buffer with DNAase I or DNAase II (Sigma Chemical Co., Poole, Dorset, U.K.) under the conditions described in the figure legends. Digestions with DNAase I were performed at pH 7.4, but for DNAase II the pH was reduced to 6.4. Aliquots were removed from the digests at different times for the determination of specifically bound radioactive steroid, as described above, and for the measurement of DNA retained on the filters by the method of Burton (16).

RESULTS

Incubation of rat thymocytes with 20 nM [1,2,4-^{3}H] triamcinolone acetonide for 1 h at 37°C gave levels of specific binding that were close to the saturation level. Binding measured by filter assay during hypo-osmotic cell lysis at

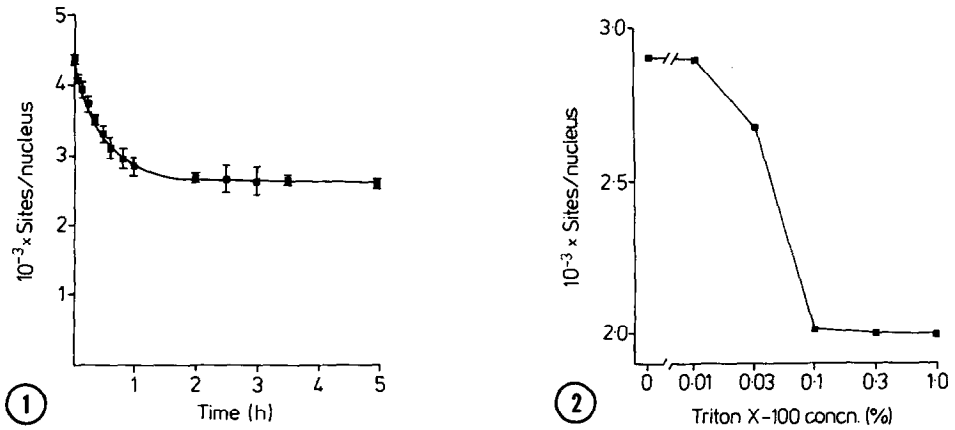

Fig. 1. Loss of bound [1,2,4-^{3}H]triamcinolone acetonide during preparation of nuclei. Cells were diluted into 25 vol. 2 mM $MgCl_2$, at 0^0C and specifically bound steroid was determined by filter assay at the times shown. Results are the mean ± 1 S.D. of 5-8 separate experiments.

Fig. 2. Extraction of nuclear-associated glucocorticoid-receptor complexes with Triton X-100. Nuclei prepared by hypo-osmotic lysis for 60 min were resuspended in STKM buffer containing various concentrations of Triton X-100. After extraction for 5 min at 0^0C, specifically bound steroid retained by the nuclei was determined by filter assay.

0^0C fell from an initial value of approx. 4500 sites/cell to a stable level of 2600-2800 sites/nucleus over a period of 1 h (Fig. 1), although cell lysis was complete within 15 min (results not shown). Nuclei containing only stably-associated glucocorticoid-receptor complexes were treated with a range of concentrations of the non-ionic detergent Triton X-100 (Fig. 2), to remove adherent cytoplasmic tags and to solubilize the nuclear membrane. Treatment with 0.1% Triton X-100 extracted 20-30% of stably bound steroid-receptor complexes; those remaining were resistant to extraction with higher concentrations of detergent. The complexes (approx. 2000/ nucleus) remaining in nuclei that had been treated in this manner were completely stable for at least 3 h at 0^0C.

The location of the detergent-resistant class of stable steroid-receptor complexes was investigated by digestion of

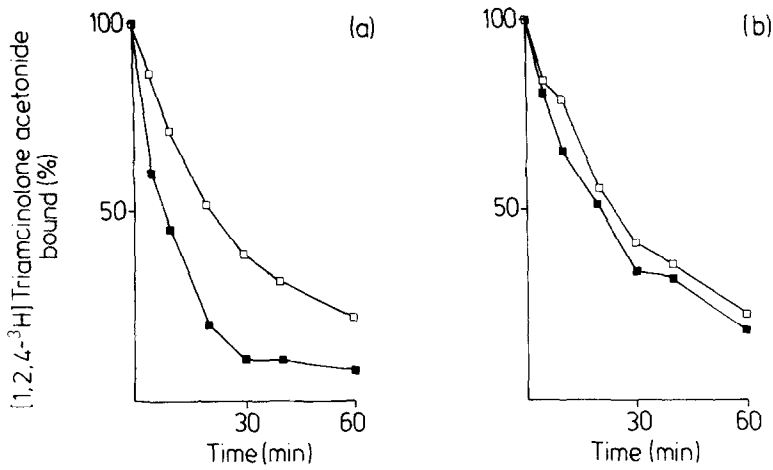

Fig. 3. Solubilization of detergent-resistant nuclear glucocorticoid-receptor complexes by DNAases I and II at 37°C. Purified nuclei (2 x 10^7/ml) were incubated at 37°C, (a) in the presence (■) and absence (□) of DNAase I (1500 Kunitz units/ml), and (b) in the presence (■) and absence (□) of DNAase II (1500 Kunitz units/ml). DNA digestion measured at 60 min was 91-95% in both cases.

Triton-washed nuclei with DNAases I and II. DNAase I was more effective than DNAase II in solubilizing nuclear glucocorticoid-receptor complexes at both 0°C and 37°C. Studies undertaken at 37°C were complicated by dissociation of steroid from the receptor (Fig. 3). At this temperature, DNAase I released approx. 95% of bound steroid (Fig. 3a) under conditions where 90-95% of DNA was digested. While the final loss of bound steroid was only 15% greater than that due to dissociation alone, the initial rate of loss was much greater in the presence of DNAase I than in its absence. Under the same digestion conditions there was no appreciable release of steroid using DNAase II beyond that due to dissociation alone (Fig. 3b).

At 0°C, where no dissociation or loss of steroid from purified nuclei was observed over the digestion period used, DNAase I solubilized 50-55% of the bound steroid-receptor complexes with digestion of only 10% of DNA (Fig. 4a), and

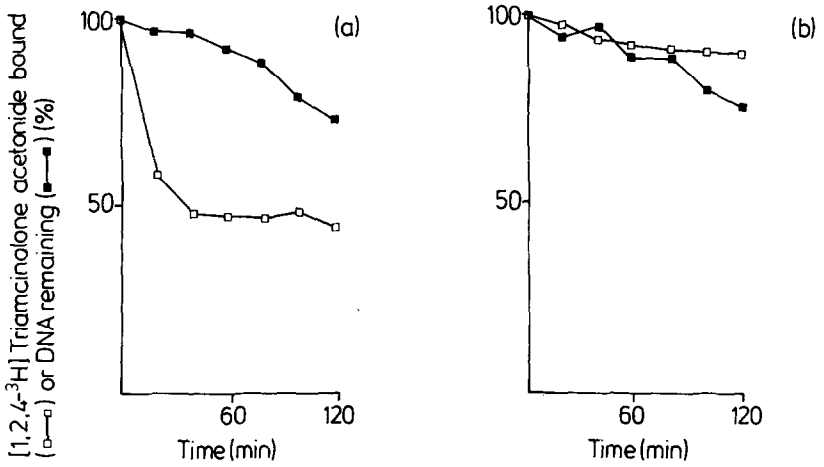

Fig. 4. Solubilization of detergent-resistant nuclear glucocorticoid-receptor complexes by DNAases I and II at 0°C. Purified nuclei (2 x 10^7/ml) were incubated at 0°C with (a) DNAase I (5000 Kunitz units/ml), and (b) DNAase II (5000 Kunitz units/ml). Samples were removed at the times shown for the measurement of DNA remaining (■) and of specifically bound steroid (□).

receptor solubilization did not increase with further digestion of DNA up to 30%. Although digestion with DNAase II gave similar results in terms of the extent of solubilization of DNA, more than 90% of the steroid-receptor complexes were resistant to digestion (Fig. 4b).

DISCUSSION

The characterization of glucocorticoid-receptor complex binding by rat thymocyte nuclei has often been performed on nuclei prepared by hypo-osmotic lysis for 15-30 min at 0°C (8,13,17). In this study we have shown that nuclear binding is partially unstable under these conditions and that loss of steroid-receptor complexes continues for 60 min, to leave a stable level of binding of 2600-2800 sites/nucleus. Since cell lysis is complete within 15 min, it is likely that all the steroid-receptor complexes remaining after this time are associated with the nucleus, and that the further loss of bound steroid is due to the loss of weakly bound nuclear

complexes. Munck and Foley (18) have shown that rat thymocytes yield two classes of transformed glucocorticoid receptors (Ia and Ib) which differ in their affinities for DNA-cellulose (Ib > Ia); it is possible that their class Ia complexes are involved in the second phase of dissociation observed here.

The stable nuclear-associated glucocorticoid-receptor complexes appear to be heterogeneous in their distribution; 20-30% of them are lost after detergent treatment of nuclei, and presumably are associated with the nuclear membrane. It is likely that the complexes remaining after this treatment are bound to specific acceptor sites within nuclear chromatin and include those responsible for regulating the physiological actions of glucocorticoids, as is the case for progesterone receptors (19). Different categories of nuclear glucocorticoid-receptor complexes in rat thymocytes have also been distinguished in terms of sensitivity to extraction with KCl; Cidlowski and Munck (8) have reported that a class of receptor complexes resistant to extraction with 0.4M KCl resides within the nucleus, whereas salt-extractable complexes are associated with the nuclear membrane.

To investigate the localization of detergent-resistant receptor complexes within chromatin, we have used DNAases I and II, endonucleases with distinct chromatin substrate specificities (20,21). The preferential release of a substantial proportion (50-55%) of steroid-receptor complexes by DNAase I under conditions where only 10% of the nuclear DNA is degraded implies that these complexes are not randomly spaced along DNA, but are selectively associated with domains of transcriptional activity. These results contrast markedly with those of Cidlowski and Munck (7,13), who found DNAase II to be more effective than DNAase I in solubilizing nuclear receptors.

However, Taira and Terayama (9) have shown that DNAase I preferentially solubilizes glucocorticoid-receptor complexes from the nuclei of AH-130 and Fu5 hepatoma cells whereas micrococcal nuclease, an enzyme with a substrate specificity similar to that of DNAase II (22), does not. Furthermore, investigations of the distribution of glucocorticoid-receptor complexes in C3H mouse mammary tumour cells have shown that these complexes appear to be concentrated in regions of active chromatin (14). Differences in the effects of nuclease digestion on receptor binding may perhaps reflect differences in methodology; studies of receptor binding in vitro to nuclease-extracted chromatin fragments of identifiable composition may clarify this issue.

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