Effects of protease inhibitors on nuclear binding of glucocorticoid hormones in C3H10T$^1/_2$ cells

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We have measured incorporation of the glucocorticoid hormone cortisone into nuclear hormone-receptor complexes in the C3H10T$^1/_2$ cell line. As we had found cortisone to be capable of malignantly transforming these cells in vitro, and certain protease inhibitors have been shown to suppress transformation in this cell line, we investigated the effects of these protease inhibitors (antipain, chymostatin and the Bowman-Birk inhibitor) on the formation of nuclear cortisone-receptor complexes. All 3 inhibitors were found to suppress wholly or partially formation of nuclear cortisone-receptor complexes, suggesting that such complexes may be involved in the process of glucocorticoid-enhanced transformation.

Glucocorticoid hormone *Protease inhibitor* *Malignant transformation* *Nuclear binding* *Hormone-receptor complex*

1. INTRODUCTION

Glucocorticoid hormones have been found to influence carcinogenesis both in vivo [1–9] and in vitro [10,11]. Although a wide variety of experimental conditions have been employed, the majority of results have reported suppressive effects of glucocorticoids on tumorigenesis [2,4,8–10], the extent of such suppression being roughly parallel to the anti-inflammatory strength of the hormones [4,8,9] as well as to their ability to inhibit DNA synthesis [9]. The relatively weak glucocorticoid cortisone, however, was found to exhibit little or no suppression of tumorigenesis in many of the above systems [2,4,8], and cortisone has been shown in other studies to increase chemically-induced tumor incidences in rodents in a co-carcinogenic or promotor-like manner [1,3,5–7]. Our in vitro studies using the C3H10T½ cell line have found that cortisone both induces malignant transformation when added alone and enhances the yield of X-ray-induced transformation [11]. We found the potent glucocorticoid dexamethasone, on the other hand, to be ineffective in transforming C3H10T½ cells either alone or in combination with X-irradiation [11].

These studies were designed to investigate the processing of cortisone and dexamethasone by C3H10T½ cells through determination of whether these hormones are incorporated into nuclear hormone-receptor complexes, and whether any such incorporation is affected by the presence of certain protease inhibitors which have the ability to suppress carcinogen-induced malignant transformation in C3H10T½ cells. Our studies on protease inhibitors as transformation-suppressing agents have been published elsewhere [12,13].

2. MATERIALS AND METHODS

C3H10T½ cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 in air, in Eagle's basal medium (BME) supplemented with 10% fetal calf serum and gentamycin (25 μg/ml). For binding experiments, cells were plated into 150-mm dishes and grown to the monolayer stage. Cells were then scraped from the plates into BME using a rubber policeman, and dispersed by

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repeated pipetting. Cell pellets were obtained by centrifugation and washed again with BME. The resulting cell pellets were dispersed in BME and incubated at 37°C with the desired radiolabelled hormone (10^{-7} M [^{3}H]cortisone, prepared through custom synthesis by New England Nuclear, 28.6 Ci/mmol; 5×10^{-9} M [^{3}H]dexamethasone, 87.0 Ci/mmol, New England Nuclear). The protease inhibitors antipain (Sigma), chymostatin (US-Japan Cooperative Cancer Research Program) and the Bowman-Birk inhibitor, prepared by us as described [12], were used at concentrations known to be effective in suppressing in vitro transformation [12,13], and were present during the full period of incubation with the hormone. Antipain was added at a concentration of 50 μg/ml, chymostatin at 0.5 or 10^{-6} μg/ml and the Bowman-Birk inhibitor at 100 or 300 μg/ml. In experiments designed to measure protease inhibitor effects on hormone binding following X-irradiation, cells were irradiated (400 rad) in the dishes immediately prior to scraping and subsequent incubation as described above. Half the irradiated samples were incubated with cortisone alone, and half with cortisone supplemented with antipain (50 μg/ml).

Following cellular incubation with hormone in the presence or absence of protease inhibitors, the incubation mixtures were diluted with 10 × volume of Earle's balanced salt solution (EBSS) at 4°C and centrifuged at 1000 × g for 5 min. (This and all subsequent steps were carried out at 4°C unless otherwise indicated.) The cell pellets were washed once with EBSS and the cells lysed through suspension in buffer A (10 mM Tris, pH 8; 0.1 M KCl; 0.5% Triton X-100; 20% (v/v) glycerol [14,15]). The lysis mixtures were centrifuged at 1000 × g for 5 min, and the resulting nuclear pellets washed once with buffer B (10 mM Tris, pH 8; 0.1 M KCl; 20% (v/v) glycerol) and once with buffer C (10 mM Tris, pH 8; 1 mM $CaCl_2$; 20% glycerol). The final nuclear pellets were dispersed in 1 ml buffer C and deposited on Gelman type A/E filters (25 mm) under suction. The incubation tubes were washed twice with 1 ml buffer C and the washings poured through the filters. 5 ml buffer C containing 1% Triton X-100 and 7% ethanol were then deposited in each filter well and left on the filter for 5 min before being sucked through. The filters were then washed continuously with 20 ml buffer C containing 1% Triton X-100. These washing solutions have been shown to be effective in removing non-specifically bound nuclear steroid [16]. The filters were then air-dried, added to 10 ml toluene-based scintillation fluid (Omnifluor, New England Nuclear) and counted (40% efficiency) in a Beckman LS-233 scintillation counter. Following scintillation counting, the filters were removed from the counting vials, air-dried and nuclear DNA assayed by the diphenylamine reaction of Burton [17] as modified by Giles and Meyers [18], using calf thymus DNA as a standard. Levels of specifically bound hormone were calculated by subtracting binding in incubation mixtures containing excess added unlabelled hormone (non-specifically bound label) from binding in incubation mixtures containing labelled hormone only (non-specifically bound plus specifically bound label).

3. RESULTS

Table 1 presents levels of nuclear uptake of [^{3}H]cortisone in C3H10T½ cells after an incubation period of 1 h (found in preliminary experiments to yield maximum cortisone uptake, not shown). It can be seen that the protease inhibitors antipain and chymostatin (0.5 μg/ml) completely suppress cortisone binding, while the Bowman-Birk inhibitor reduces cortisone binding by approx. 56%. A considerably lower concentration of chymostatin (10^{-6} μg/ml, a concentration sufficient to inhibit radiation-induced transformation in C3H10T½ cells [13]), is ineffective in inhibiting nuclear cortisone binding. Irradiation of the cells followed by cortisone incubation in the presence or absence of antipain resulted in the elimination of hormone binding. In experiments conducted analogously to cortisone-binding experiments, neither antipain (50 μg/ml), chymostatin (50 μg/ml), nor Bowman-Birk inhibitor (300 μg/ml) was found to affect nuclear uptake of [^{3}H]dexamethasone in C3H10T½ cells (J. Carew, unpublished).

4. DISCUSSION

These experiments were designed to investigate putative glucocorticoid binding to nuclei of C3H10T½ cells, and to ascertain whether any such

Table 1

Effects of protease inhibitors and of pre-irradiation on nuclear binding of cortisone in C3H10T½ cells

Treatment	Binding (pmol cortisone/mg DNA)	
	+ treatment	− treatment
Antipain (50 μg/ml)	−0.014 ± 0.015[a]	0.106 ± 0.023
Chymostatin (0.5 μg/ml)	−0.027 ± 0.030[a]	0.105 ± 0.045
Chymostatin (10^{-6} μg/ml)	0.076 ± 0.007	0.079 ± 0.033
Bowman-Birk inhibitor (100 μg/ml)	0.024 ± 0.007	0.055 ± 0.009
Pre-irradiation (400 rad)[b]	0.002 ± 0.021	−0.028 ± 0.022[a]

[a] Hormone binding values are obtained as the numerical difference of 2 independent measurements (binding levels in incubation mixtures containing labelled plus unlabelled hormone subtracted from binding levels in incubation mixtures containing labelled hormone only. See section 2). Thus a system in which zero binding is occurring may exhibit a mean of measurements falling on the negative as well as the positive side of zero. All 'negative' means have been found by the Wilcoxen (Mann-Whitney) ranking test not to differ significantly from zero

[b] The '+ treatment' value for 400 rad pre-irradiated cells arises from post-irradiation incubation of the cells with cortisone in the presence of antipain. The '− treatment' value arises from post-irradiation incubation with cortisone alone

Results represent the mean ± SE of 8 separate determinations (antipain and Bowman-Birk inhibitor) and 4 separate determinations (chymostatin and antipain preceded by 400 rad irradiation). Treated and untreated samples were found to be statistically different (using Student's *t*-test for independent samples) in the case of antipain ($p<0.001$), chymostatin, 0.5 μg/ml ($p<0.05$), Bowman-Birk inhibitor ($p<0.02$). Other treatments were not found to affect binding in a statistically significant manner ($p>0.05$)

binding is affected by the presence of protease inhibitors. Protease inhibitors have been shown to suppress transformation induced by radiation [12,13], chemicals [19,20], the sex hormone estradiol [21] and the glucocorticoid hormone cortisone ([11] and unpublished). It was our intention to examine a common and much-studied feature of steroid hormone action, binding of hormone to a specific receptor protein, to determine whether such binding was exhibited by glucocorticoids in transformable C3H10T½ cells and whether any such binding was affected by protease inhibitors capable of suppressing glucocorticoid-induced transformation. Our results indicating that 3 protease inhibitors inhibit formation of nuclear cortisone-receptor complexes suggest that hormone-receptor complex formation may play a role in cortisone-induced transformation. These results are consistent with our earlier finding that antipain both inhibited estradiol-induced transformation in C3H10T½ cells [21] and inhibited nuclear uptake of labelled estradiol in the MCF-7 breast tumor cell line [22], but this is the first report of protease inhibitors suppressing nuclear steroidal uptake (table 1) in a cell system in which they are also capable of suppressing steroidal-induced transformation.

It is of interest that antipain and chymostatin exhibit complete suppression of cortisone binding in this system, while the Bowman-Birk inhibitor exhibits partial suppression. This may be due to the relative sizes of these inhibitors. The smaller peptide-like substances antipain and chymostatin are likely to be capable of entering the cell and thus could directly perturb hormone-receptor binding events (such as the putative proteolysis of the native glucocorticoid receptor to convert it from large, non-steroid-binding forms to smaller forms capable of binding steroid [23,24], or direct receptor binding of steroid [25,26]). The considerably larger Bowman-Birk inhibitor is unlikely to penetrate the cell membrane [12] and thus may exert its effect through a more remote, or indirect, mechanism.

The effects of pre-irradiation in temporarily eliminating nuclear cortisone binding in the presence or absence of antipain (table 1) do not necessarily argue against a role for hormone-receptor complexes in cortisone-enhanced radiation transformation. In these experiments, cortisone was present for only 1 h post-irradiation while in previously reported transformation experiments the hormone was applied at frequent intervals and for an extended period post-irradiation [11]. We have recently observed that cortisone has its enhancing/promotional effect on the transfor-

mation process when given during the expression period of radiation transformation, or at long time periods post-irradiation [27]. Further experiments involving measurement of cortisone-receptor binding levels following repeated applications of the hormone post-irradiation will be necessary to test whether the hormone-receptor complex may be involved in the hormone enhancement of radiation-induced transformation.

In summary, the results presented here are compatible with a role for the cortisone-receptor complex in cortisone-induced transformation of C3H10T½ cells [11]. The mechanism through which glucocorticoid-receptor complexes could contribute to the transformation process is not yet clear, and must await a more thorough characterization of molecular events resulting from glucocorticoid activity in this cell system.

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