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GLUCOCORTICOID EFFECTS AND RECEPTORS IN TWO RAT COLON CARCINOMA CELL LINES DIFFERING BY THEIR TUMORIGENICITY

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Summary—Steroid hormones, regulators of cell differentiation and proliferation, are believed to play a role in carcinogenesis. Glucocorticoid hormones in particular modulate the expression of a number of proteins implicated in this process. We have investigated the effect of dexamethasone on two cell lines derived from a colon carcinoma, which differ by their tumorigenicity. Dexamethasone was found to inhibit growth of both the progressive (PROb) and the regressive clone (REGb). Upon hormonal treatment, glucocorticoid hormones induced fibronectin secretion by the two clones, whereas PROb cells were found to secrete an additional $M_r \sim 43{,}000$ protein. The cellular effect of glucocorticoid hormones being mediated through a specific soluble receptor, we have characterized this protein. The progressive cells (PROb) contained more specific glucocorticoid-binding sites ($\sim 170{,}000$ sites per cell) than the regressive ones (REGb cells; $\sim 100{,}000$ sites per cell). In both clones, the receptor was associated with the $M_r \sim 90{,}000$ heat shock protein to yield large complexes (Stokes radius $R_s \sim 7.5$ nm), which were dissociated to the same extent upon heat- and salt-treatment. The steroid- and DNA-binding unit of the receptor, characterized under denaturing conditions using an anti-receptor monoclonal antibody was found to be more degraded in the progressive cell line.

INTRODUCTION

Steroid hormones are known to affect the proliferation, the metabolism, and the differentiation of both normal and malignant cells [1–3]. These effects of steroid hormones are mainly mediated by specific receptors, and it is now well established that the presence or absence of steroid receptors in malignant cells is of importance in the regulation of tumor growth. For instance, the presence or absence of estrogen and progesterone receptors is an accurate predictor of responsiveness to endocrine therapy in patients with breast cancer [4, 5]. In colonic tumors, the presence of receptors for estrogen [6–8], progesterone [6–8], androgen [9, 10], mineralocorticoid [11] and glucocorticoid hormones [6] has been clearly demonstrated.

Glucocorticoid hormones in particular have been shown to influence a wide variety of different cell properties. They generally have an antiproliferative effect [12–15], although growth-stimulatory responses have been seen in foreskin fibroblasts [16] and in a human leiomyosarcoma cell line [17]. They also appear to be involved in different steps of carcinogenesis and formation of metastasis, as they up-regulate the production of different proteins which are involved in these processes, such as fibronectin [18–20] and a number of proteases [21].

All these observations suggest that glucocorticoid hormones may play an important role in the biology of cancer. The purpose of this study was to investigate the effect of glucocorticoid hormones on two rat colon carcinoma cell lines differing by their tumorigenicity: after s.c. injection into syngeneic BDIX rats, the cell line REGb yields regressive tumors, while PROb cells give progressive tumors which spread into the lungs, the kidneys, and the lymph nodes [22–24]. We also performed a detailed characterization of the glucocorticoid receptors present in these two cell lines.

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Abbreviations: Dexamethasone, 9α-fluoro-16α-methyl-11β,17α,21-trihydroxy-pregna-1,4-diene-3,20-dione; FCS, fetal calf serum; hsp90, $M_r \sim 90{,}000$ heat shock protein; MOPS, 3-(*N*-morpholino)propanesulfonic acid; SDS–PAGE, polyacrylamide gel electrophoresis performed in the presence of sodium dodecyl sulfate; SSC, standard saline citrate (0.15 M NaCl, 0.015 M sodium citrate, pH 7.4).

EXPERIMENTAL

Materials

RPMI 1640, fetal calf serum, glutamine, and trypsin–EDTA were from GIBCO (Grand Island, N.Y.). [1,2,4-(*n*)-^{3}H]Dexamethasone (45 Ci/mmol), [methyl-^{3}H]thymidine (20 Ci/mmol) and [^{35}S]methionine (800 Ci/mmol) were obtained from the Radiochemical Centre (Amersham, England). Unlabeled dexamethasone was from Roussel–Uclaf (Romainville, France). Amplify™ and Hyperfilm-MP were from Amersham. Monoclonal mouse anti-glucocorticoid receptor antibodies were obtained from Dr J.-Å. Gustafsson and A.-C. Wikström (No. 7, see Ref. [25]). Affinity purified polyclonal rabbit anti-hsp90 antibodies were prepared using purified rat liver hsp90 as immunogen and have been characterized previously [26]. All other chemicals were analytical grade products from Sigma (St Louis, Mo.).

Cell culture

PROb and REGb rat colon adenocarcinoma cell lines (gifts from Dr F. Martin) were grown in complete medium (RPMI 1640, 2 mM L-glutamine) supplemented with 10% heat inactivated fetal calf serum depleted for steroids by incubation at 4°C for 2 h with dextran-coated charcoal (5% Norit A, 0.5% Dextran T70). For labeling experiments, cells were grown in methionine-free MEM medium supplemented with 2 mM L-glutamine and 5 μCi of [^{35}S]methionine per ml of medium. To determine the effect of glucocorticoid hormones on growth, cells were seeded at a density of 5×10^4/ml in 24-well culture plates. Stock solutions of steroids were prepared in ethanol, and added to the medium so that the final ethanol concentration never exceeded 0.1%. Control cultures were incubated with the same concentration of vehicle. Studies were performed in quadruplicate. Cell growth was estimated on the basis of [^{3}H]thymidine incorporation (0.5 μCi/ml) for 4 h at 37°C.

Ligand binding assays

Preliminary kinetic studies were performed to define the equilibrium-binding modalities. In this analysis, cells were incubated for different times (15 min to 7 h) with 1 nM [^{3}H]dexamethasone in the presence or absence of a 50-fold excess of unlabeled ligand. Under our experimental conditions, the equilibrium was reached after 45 min (not shown). In Scatchard plot analyses, various concentrations (1–25 nM) of [^{3}H]dexamethasone were used. Non-specific binding was determined by incubating cells in the presence of a 50-fold excess of unlabeled dexamethasone. After incubation for 60 min, culture medium was removed, adherent cells were washed three times with 1.5 ml cold PBS then lysed with 0.2 ml of 0.1 N NaOH. Lysates were collected and assayed for radioactivity.

Cytosol preparation

Cells were homogenized in PEG (20 mM sodium phosphate, 1 mM EDTA, 10% (v/v) glycerol, 10 mM mercaptoethanol, 0.2 mM PMSF, 2% (v/v) acetonitrile, 1 mM bacitracine, 5 mM benzamidine, pH 7.4) or in PEGM buffer (20 mM sodium molybdate in PEG buffer) using a Teflon–glass Potter homogenizer. Centrifugation was performed as described [27]. The resulting supernatant was used immediately, or stored at −70°C until further use.

SDS–PAGE and protein immunoblot

Preparation of samples and polyacrylamide gel electrophoresis were as previously detailed [26]. Following electrophoresis, gels were fixed in 7% acetic acid for 30 min and impregnated with Amplify™. The gels were then dried under vacuum and exposed to Hyperfilm-MP at −70°C. For immunoblotting experiments, proteins separated by SDS–PAGE were transferred to nitrocellulose membranes [26]. The $M_r \sim 90{,}000$ heat shock protein and the glucocorticoid receptor were detected using affinity-purified rabbit antibodies [26] or the monoclonal anti-receptor antibody No. 7 [25], respectively.

High performance size exclusion chromatography

Glucocorticoid–receptor complexes were analyzed by high performance size exclusion chromatography on a Superose 12 column (Pharmacia, Sweden). Elution was performed at a flow rate of 0.35 ml/min with PEM buffer (PEGM without glycerol) supplemented with 0.15 M NaCl. 1-min fractions were collected and assayed for radioactivity. Calibration was carried out with β-galactosidase ($R_S \sim 6.9$ nm), aldolase ($R_S \sim 4.8$ nm), bovine serum albumin ($R_S \sim 3.55$ nm) and myoglobin ($R_S \sim 2.0$ nm). Void volume and total volume were estimated with blue dextran 2000 and [^{3}H]H_2O, respectively. The distribution coefficients were calculated from $K_d^{1/3}$ vs R_S according to Porath [28].

Separation of glucocorticoid–receptor complexes

The extent of transformation was determined by measuring the DNA-binding activity of glucocorticoid–receptor complexes, using a modification [27] of the procedure described by Holbrook *et al.* [29].

RNA preparation and detection of glucocorticoid receptor RNA transcripts

Total cellular RNA was extracted from 530 cm^2 plates of confluent cells using the guanidine thiocyanate method [30]. 30 μg of total RNA were combined with 5 μl deionized formamide, 1.5 μl formaldehyde, and 1 μl of 10× MOPS buffer [31] heated at 68°C for 5 min, and then cooled on ice. The samples were loaded onto a 1% agarose minigel containing 2.2 M formaldehyde and electrophoresed for 1 h at 100 V. The RNA was transferred to Amersham Hybond-N. Following transfer, RNA was crosslinked to the membrane by u.v. irradiation for 5 min [32]. The amount of RNA per lane on the filters was judged to be equal by ethidium bromide staining of the ribosomal bands (not shown). The nylon filter was prehybridized for 6 h at 65°C in 2× SSC, 1% SDS, 10% dextran sulfate, 1 M NaCl. Hybridization was performed for 20 h at 65°C in the same solution containing 10^6 cpm/ml of a 2.2 kb fragment of the rat glucocorticoid receptor cDNA (Ref. [33]; plasmid pRdN93 kindly provided by Dr K. Yamamoto and R. Miesfeld) labeled by random priming. Following hybridization, the membrane was washed with 2× SSC at room temperature, 1× SSC and 1% SDS at 65°C, 0.5× SSC at room temperature, and then exposed for 2 days to an Hyperfilm-MP.

Miscellaneous

Quantification of proteins was performed according to the method of Bradford [34] using bovine serum albumin as standard. Radioactivity was measured in a Beckman LS 2800 liquid scintillation counter with a tritium efficiency of 40–50%.

RESULTS AND DISCUSSION

Growth inhibition by dexamethasone

The effect of different concentrations of dexamethasone on the growth of PROb and REGb cells in medium supplemented with 10% FCS is illustrated in Fig. 1. Dexamethasone induced a dose-dependent decrease in cell proliferation as determined by [^{3}H]thymidine incorporation. Under these experimental conditions, significant inhibitory effects on the growth of both cell lines were observed 12 h after the addition of 10^{-7}–10^{-6} M dexamethasone. Similar curves were obtained with different concentrations of serum in the medium (data now shown). In all cases, the inhibitory effect was higher for REGb cells as compared to PROb cells.

For both clones, this inhibitory effect on growth could not be related to an increased cell death as very small amounts of proteins were recovered in the culture supernatants (cf. Fig. 2). We also compared the aspect of the cells following treatment with dexamethasone, but no striking morphological difference was noted between control cultures and hormone-treated cells (data not shown).

Effect of dexamethasone on protein secretion

Cytosolic extracts prepared from cells grown for 12, 24 or 36 h in medium supplemented with

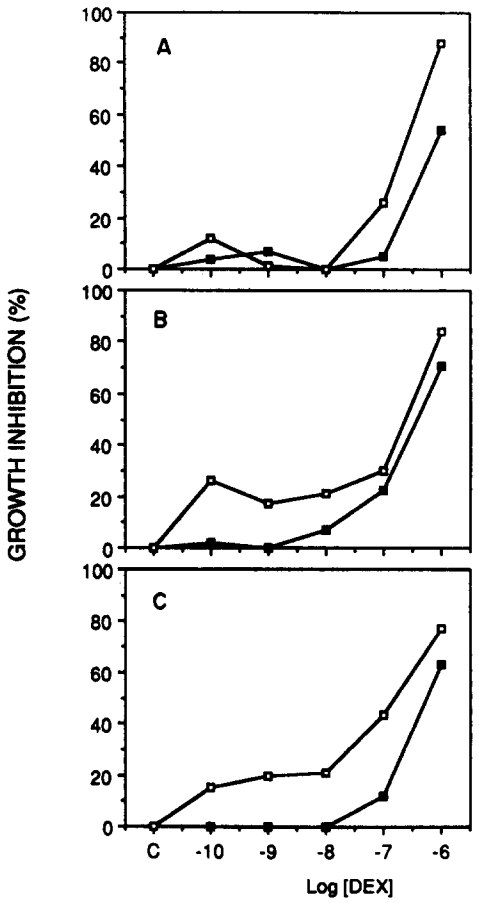

Fig. 1. Glucocorticoid regulation of growth. PROb (■) and REGb (□) cells were grown in complete medium supplemented with different concentrations of dexamethasone for 24 h (A), 48 h (B) or 72 h (C). Growth inhibition was monitored by [^{3}H]thymidine incorporation as described in "Experimental". Average values from quadruplicate assay points are indicated.

[^{35}S]methionine in the presence or absence of 10^{-6} M dexamethasone were analyzed by SDS gel electrophoresis followed by autoradiography. No difference was seen when the pattern of cytosolic proteins were compared (data not shown). However, when culture supernatants from these labeling experiments were analyzed under the same conditions, we found that the secretion of an $M_r \sim 220{,}000$ protein by PROb and REGb cells was significantly induced by dexamethasone (Fig. 2). This protein has been identified as fibronectin (data not shown), the synthesis of which is known to be up-regulated by glucocorticoids [18–20]. However, the enhanced induction was slightly different between the two cell lines, as determined by densitometric scanning analysis of the autoradiograms. We calculated that dexamethasone induced a ~10-fold increase in fibronectin secretion by PROb cells, but only a ~5-fold increase for REGb cells. This result is in complete agreement with immunostaining of these cells following hormonal treatment using anti-fibronectin antibodies (M. Gregoire, personal communication).

Two additional changes on the pattern of protein secretion can be seen on Fig. 2. The synthesis of an $M_r \sim 40{,}000$ protein by REGb cells seems to be repressed by dexamethasone. In fact, this band was not always seen, and corresponded to a major cellular protein released from dead cells. In contrast, the progressive cells secreted an $M_r \sim 43{,}000$ protein when the medium was supplemented with dexamethasone. Interestingly, an $M_r \sim 43{,}000$ protein has been found to be induced by glucocorticoids in a variety of cells and tissues [35]. Whether these proteins are identical remains to be determined.

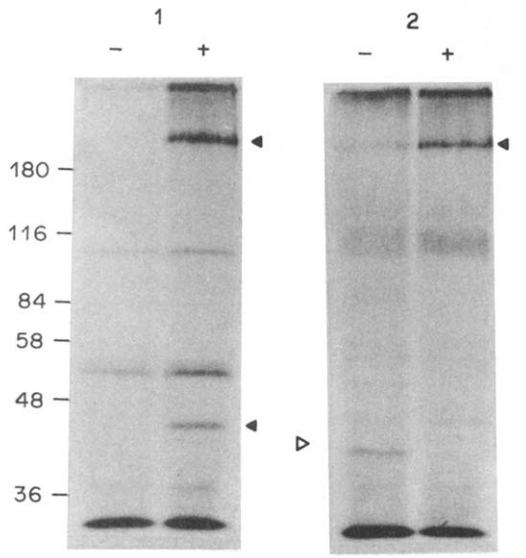

Fig. 2. Modulation of protein secretion by dexamethasone. The media of actively growing PROb cells (1) and REGb cells (2) were replenished with fresh serum-free media containing [^{35}S]methionine (5 μCi/ml) and 10^{-6} M dexamethasone (+) or [^{35}S]methionine without dexamethasone in a parallel set of culture wells (control; −). [^{35}S]Methionine-labelled secreted proteins in the media (1 ml) were processed for SDS–PAGE as described under "Experimental". Position of standard proteins are indicated on the left side (molecular weights × 10^{-3}).

As most of the effect of glucocorticoids are mediated through a soluble receptor protein, it was of interest to investigate the glucocorticoid receptors in these two cell lines, and to compare them using experiments involving quantitative as well as qualitative characteristics.

Glucocorticoid receptor-binding assays

We first determined the number of glucocorticoid binding sites in the two clones using a whole cell binding assay. When the data were plotted according to Scatchard [36], a straight line was obtained for both clones, suggesting a single class of binding sites. In Fig. 3 is shown the result of a typical experiment. The means and standard deviations of 6 experiments are summarized in Table 1.

When cytosol was used instead of total cells and incubated with increasing concentrations of [^{3}H]dexamethasone, similar results were found (data not shown) indicating that the difference in dissociation constant and number of sites (~70 fmol per mg protein for PROb cells and ~35 fmol per mg protein for REGb cells) between PROb and REGb cells could not be related to a difference in uptake of ligand by the cells. The parameters determined are in the range of the values reported for experimental colonic tumors [13, 37] and human colon

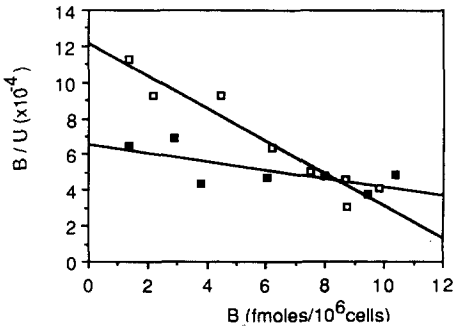

Fig. 3. Binding of [^{3}H]dexamethasone to receptors in progressive and regressive clones. Specific binding of [^{3}H]dexamethasone to glucocorticoid receptors of clones PROb (■) and REGb (□) was determined in whole cells as described in "Experimental". Binding was determined over a concentration range of 1–25 nM [^{3}H]dexamethasone and results were plotted by the method of Scatchard.

Table 1. Summary of glucocorticoid binding characteristics in PROb and REGb cells

	B_{max} sites/cell	K_d $10^9\ M^{-1}$
PROb cells	166,850 ± 31,160	0.024 ± 0.007
REGb cells	97,575 ± 33,970	0.091 ± 0.014

cancers [6]. In addition, the number of glucocorticoid binding sites has been found to be larger in tumors than in normal colon [37], which is in agreement with our demonstration that the progressive cell line contains more receptors than the regressive clone.

The enhanced transcription of a reporter gene has recently been correlated with the amount of receptor present in the sample [38]. The induction of fibronectin synthesis was higher in PROb cells than in REGb cells. Consequently, it is tempting to correlate this difference with the higher number of glucocorticoid binding sites detected in PROb cells. But if this is the case, then the more potent effect on growth inhibition of REGb cells suggests that glucocorticoids can inhibit the synthesis of growth factor(s) involved in the autocrine stimulation of cell division/proliferation. A candidate for modulating growth of PROb and REGb cells could be the $M_r \sim 30{,}000$ protein with glucocorticoid suppressible mitogenic activity recently characterized by Cook *et al.* [39].

Glucocorticoid receptor–hsp90 interaction

In crude cytosolic extracts, the glucocorticoid receptor is associated with a dimer of the $M_r \sim 90{,}000$ heat shock protein, hsp90 [40–42]. Recently, the ability of the glucocorticoid receptor to interact with its ligand has been correlated to the receptor–hsp90 interaction [43, 44]. Therefore we decided to assess whether the difference in glucocorticoid binding sites measured was due to a difference in ligand-binding ability, or whether this was related to a difference in amount of receptor expressed in the two clones. We first compared the amount of hsp90 present in cytosol prepared from the two cell lines, using specific polyclonal antibodies to probe western immunoblots. As illustrated in Fig. 4, the amount of hsp90 expressed in the two clones was not significantly different. hsp90 was found to represent ~2% of total cytosolic proteins as determined by densitometric scanning analysis of Coomassie blue stained SDS-gels (data not shown). This results is consistent with the values determined for normal tissues as well as for tumor cell lines (Ref. [26] and M. Denis, J. McGuire, A.-C. Wikström and J.-Å. Gustafsson, unpublished data).

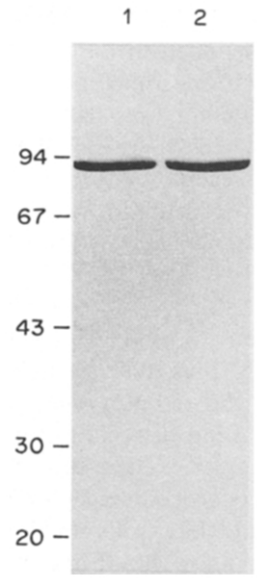

Fig. 4. PROb and REGb cytosols contain similar amounts of hsp90. Cytosols prepared from PROb cells (1) and REGb cells (2) were run on a 10% (w/v) SDS–PAGE. Following transfer of the proteins, the nitrocellulose filter was incubated with 1:50 dilution of the affinity purified anti-hsp90 antibodies, then with alkaline phosphatase-labelled anti-rabbit immunoglobulins. Positions of standard proteins are indicated on the left side (molecular weights × 10^{-3}).

In order to test the interaction of the receptor with hsp90 we analyzed glucocorticoid–receptor complexes by size exclusion chromatography. Samples incubated with a saturated concentration of ligand were loaded on a Superose 12 column equilibrated with buffer containing sodium molybdate. Fractions were collected and assayed for radioactivity. The results of a typical experiment are presented in Fig. 5. It is clear from this chromatogram that glucocorticoid-receptor complexes from both clones

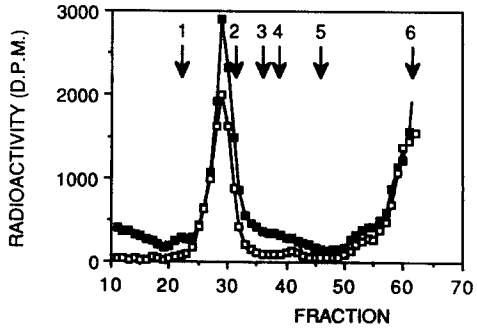

Fig. 5. High performance size exclusion chromatography of glucocorticoid receptor from PROb and REGb cytosols. Cytosol prepared from PROb cells (■) or REGb cells (□) in PEGM buffer was labelled with [^{3}H]dexamethasone. Labelled preparations were chromatographed on a Superose 12 column as described in "Experimental".

had a similar Stokes radius (~7.5 nm). Furthermore, it corresponded to the value determined under identical experimental conditions for receptor–hsp90 complexes from rat liver cytosol [45]. The conclusion, therefore, is that all the glucocorticoid–receptor complexes from both clones were associated with hsp90.

Transformation of glucocorticoid–receptor complexes

Glucocorticoid hormones are thought to control gene expression through a specific interaction of the receptor with target DNA sequences following dissociation of the receptor–hsp90 complex. Therefore, we assessed whether glucocorticoid–receptor complexes from PROb and REGb cells could be converted to a DNA-binding species. First, fixed amounts of cytosolic proteins incubated with 50 nM [^{3}H]dexamethasone were loaded on mini-columns. Table 2 shows that in the presence of sodium molybdate, a very small percentage of glucocorticoid–receptor complexes were retained on the DNA–cellulose column. Following treatment at 25°C for 30 min in the presence of 300 mM KCl and absence of molybdate, conditions known to induce the transformation of the glucocorticoid receptor [46], the percentage of complexes converted to a DNA-binding form was increased to 60–70% for both cell lines. Using this technique, we could not detect a quantitative difference in the transformation process induced *in vitro* by salt- and heat-treatment.

Analysis of glucocorticoid receptor monomer under denaturing conditions

In order to analyze the monomeric glucocorticoid binding protein, we have assayed cytosol of these cells with a monoclonal antibody that has been raised against the rat receptor [25]. Proteins, separated by SDS–PAGE, were transferred to nitrocellulose membrane, and receptor was localized by incubation of the filter with the monoclonal anti-receptor antibody followed by alkaline phosphatase labeled anti-mouse immunoglobulins antibodies. Figure 6 shows an analysis of cytosolic extracts of PROb (lane 1) and REGb (lane 2) cells. This result partially parallels ligand binding assays in that REGb cells harbor reduced glucocorticoid receptor than PROb cells that is detected by the antibodies. However, the most striking difference is that in the regressive cells, an $M_r \sim 94{,}000$ protein, expected size for the intact rat receptor [25], is detected on the membrane, whereas this band is almost not present in cytosol prepared from progressive cells. In both clones, an $M_r \sim 79{,}000$ protein was detected in similar quantities, which might correspond to a frequently observed proteolytic fragment of the receptor [47]. This suggested that the receptor protein was degraded by proteases in the cytosols. However, at this point we could not exclude that the proteins detected by the antibodies represented normal translational products of degraded mRNAs. In order to test this hypothesis, we prepared total RNA from both clones, and determined the size of the messengers encoding the receptor using 2.2 kb fragment of the rat glucocorticoid receptor [33]. As shown in Fig. 7, this probe detected a ~7 kb transcript in both clones. Although different transcription initiation sites might exist within the receptor mRNA of the two clones and explain the protein pattern observed, we found this explanation unlikely, and our conclusion,

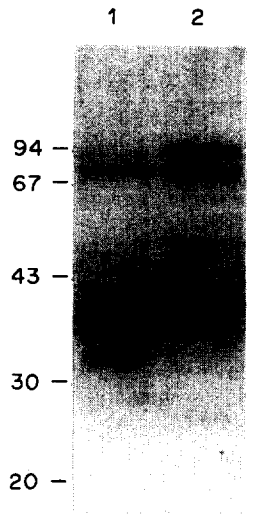

Fig. 6. Immunoblot analysis of glucocorticoid receptor in PROb and REGb cytosols. Cytosol prepared from PROb cells (1) and REGb cells (2) were run on a 10% (w/v) SDS–PAGE. Following transfer of the proteins, the nitrocellulose filter was incubated with a 1:500 dilution of the anti-glucocorticoid receptor monoclonal antibody No. 7, then with alkaline phosphatase-labelled anti-mouse immunoglobulins. Positions of standard proteins are indicated on the left side (molecular weights × 10^{-3}).

Table 2. DNA-binding properties of glucocorticoid–receptor complexes from PROb and REGb cells

Cytosol from:	Heat and salt treatment	% of binding to DNA	DEAE	HAP
PROb cells	–	8.3	89.0	2.7
	+	67.9	10.6	21.5
REGb cells	–	11.6	83.1	5.3
	+	65.7	12.1	22.2

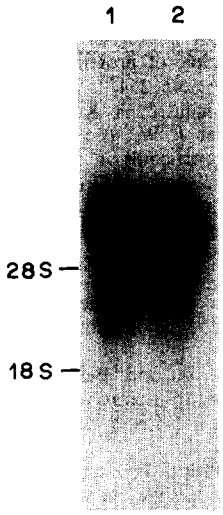

Fig. 7. Northern blot analysis of PROb and REGb RNA. Total RNA prepared from PROb cells (1) and REGb cells (2) were analyzed using a portion of the rat glucocorticoid receptor cDNA as a probe, as detailed under "Experimental". On the left side are indicated the positions of the ribosomal 18S and 28S RNA.

therefore, was that the receptor protein was more degraded in the progressive cells. A simple explanation would be that PROb cells express more proteolytic enzymes than REGb cells. This is being investigated in our department. Alternatively, the receptors from the two clones might have a slightly different aminoacid sequence, making the PROb receptor more sensitive to proteolytic degradation. Finally, we have proposed that the glucocorticoid receptor is a glycoprotein [48], and Kasbaoui *et al.* [49] have recently demonstrated that fibronectin from PROb cells is less glycosylated, and more sensitive to proteases than fibronectin secreted by REGb cells. A similar mechanism can be proposed for the glucocorticoid receptor.

In conclusion, we have studied the effect of glucocorticoid hormones on two clones isolated from a colon carcinoma which differ by their tumorigenicity. These two clones responded in a different manner to hormonal treatment. Consequently, it was of interest to analyze the glucocorticoid receptor at the protein and RNA level. We also found differences in the receptor protein between the two clones, which might be correlated with the modulation of growth and secretion observed. Thus, these two clones appear to be an interesting model in terms of tumorigenicity and glucocorticoid hormone action. A detailed study of the glucocorticoid suppressible proliferative factor(s) as well as the $M_r \sim 43{,}000$ protein secreted by PROb cells might give some new informations on the role of glucocorticoid hormones in carcinogenesis.

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