Biochemical Pharmacology, Vol. 32, No. 5, pp. 919–921, 1983.
Printed in Great Britain.

0006–2952/83/050919–03 $03.00/0

SHORT COMMUNICATIONS

Progesterone receptor in cultured mouse fibroblast L-cells

(*Received* 24 *May* 1982; *accepted* 3 *September* 1982)

Transformed mouse fibroblast L-cells offer a convenient system to investigate hormone action [1–5] as receptors for various steroids were demonstrated in the different lines. The presence of a glucocorticoid receptor [1–5] as well as androgen and estrogen receptors [4] has been reported. Progesterone receptors in L-cells, however, have not yet been identified [4, 5]. We report here that in certain mouse fibroblast L-cell variants, besides their well-known glucocorticoid receptor [2], a specific, high-affinity, saturable progesterone-binding site can be detected by both *in vivo* and *in vitro* techniques.

Materials and methods

Cell culture. Cells of the transformed mouse fibroblast L-cell line SLB8217 R^{+*} were used in these experiments. This clone was isolated originally by Lippman and Thompson [1]. Cells possess apparently normal glucocorticoid receptors but are resistant to dexamethasone [2]. Culture medium was Eagle's minimal essential medium (Gibco, U.S.A.) supplemented with 5% fetal calf serum (Flow Laboratories, U.K.). Details for culture conditions have been published previously [2].

Steroids. [1,2-*n*-^{3}H]Dexamethasone (25–38 Ci/mmole); [1α,2α-*n*-^{3}H]progesterone (49 Ci/mmole) were purchased from the Radiochemical Centre (Amersham, U.K.). The following unlabelled steroids were used: dexamethasone, cortisol, aldosterone, progesterone, 17-α-methyltestosterone, 17-β-estradiol (Sigma, U.S.A.). DES was obtained from Sigma. R5020 (promegestone) was a gift from Dr M. E. Lippman (NCI, NIH, Bethesda, MD).

Receptor assays in intact whole cells. Competition assays were carried out by a modification of the method of Sibley and Tomkins [6] as previously described [2]. Briefly, cells were grown to high density, removed by trypsin–EDTA, and washed 3 times with PBS at 4°. For measuring steroid uptake about 2×10^6 cells were resuspended in 0.2 ml serum-free culture medium containing either [^{3}H]dexamethasone or [^{3}H]progesterone in the absence or presence of the indicated excess of unlabelled competitors. After incubation for 40 min at 37° the cells were washed quickly 4 times with PBS at 4°. Cell-associated radioactivity was determined by dissolving the washed cell pellet directly in scintillation liquid (Tritosol) and counting in a Packard Tricarb liquid scintillation counter. The amount of specific binding was taken as the difference in the radioactivity between cells incubated with labelled hormone only and a parallel sample containing both labelled and non-radioactive steroids. In the presaturation experiments cells were first incubated for 20 min at 37° in 0.1 ml serum-free culture medium with or without 5 μM unlabelled steroid; thereafter the same volume was added with the indicated steroids and the procedure was conducted as described earlier. To determine the intracellular distribution of [^{3}H]progesterone the method of Sibley and Tomkins [6] was adopted.

Receptor assays in particle-free cell extracts (cytosol). Cytosolic receptor binding assays were performed according to Baxter and Tomkins [7]. Cytosol was prepared in homogenizing buffer [0.001 M Tris–HCl (pH 7.4), 0.0015 M EDTA, 0.0005 M dithiothreitol]. Cell homogenization and all subsequent steps were carried out at 0–4°. Bound radioactivity was determined after exposing the cytosol very briefly to dextran-coated charcoal (Norit A, activated), shaking and immediately centrifuging the tubes. Data were plotted according to Scatchard [8]. Protein was determined according to Lowry *et al.* [9] using bovine serum albumin as standard.

Results and discussion

To characterize the glucocorticoid- and progesterone-binding sites of the SL cells we first performed competition and cross-competition assays. Intact cells were incubated at 37° with a saturating concn of either [^{3}H]dexamethasone or [^{3}H]progesterone and with three different concns of the non-labelled competitors. Fig. 1A shows that unlabelled dexamethasone competes strongly for the [^{3}H]dexamethasone-binding sites. Progesterone and promegestone are also effective in displacing [^{3}H]dexamethasone from its binding sites in a concn-dependent manner. Recently progesterone was reported to destabilize the G–R complex [10, 11], an effect mediated by an allosteric type secondary binding site of the G–R complex. Thus the decrease in the binding of [^{3}H]dexamethasone by progesterone can be explained—at least in part—by the enhanced dissociation of dexamethasone from the G–R complex [10].

In Fig. 1B we summarize the data of the competition and cross-competition studies for the [^{3}H]progesterone binding. The assay was extended for some other steroids as well in order to get information about the specificity of the binding. As is shown only progesterone and R5020, a synthetic progestin [12], have a dose-dependent competitive activity among the steroids examined. Both dexamethasone and aldosterone, the latter a potent mineralcorticoid with light glucocorticoid activity, have only a minor effect on the binding of progesterone, whatever the excess used. Similarly estradiol and DES compete only slightly. As the observed small effect of these compounds is independent from the actual excess of the competitors, this reaction is believed to be non-specific in the sense that competitors in these cases may liberate progesterone from sites different from the true progesterone receptor. Interestingly, testosterone is the most active heterocompetitor; its effect, however, is not concn-dependent either.

Competition studies indicate the parallel existence of two separate binding sites; one of which binds preferentially glucocorticoids, but has an appreciable affinity also for progestins, and another which binds (specificially) only progestins. Since progesterone interacted with both kinds of binding sites present in the SL cells, in order to be able to investigate them separately we first presaturated the steroid-binding sites with a large excess of unlabelled dexamethasone or progesterone, respectively, and thereafter the "residual specific binding" was determined for each steroid (Table 1). Non-presaturated controls received only

* Abbreviations: SL, SLB8217 R^+ cell line; G–R, glucocorticoid–receptor; P–R, progesterone–receptor; CBG, corticoid-binding globulin; PBS, potassium phosphate buffered isotonic saline; DES, diethylstilbestrol.

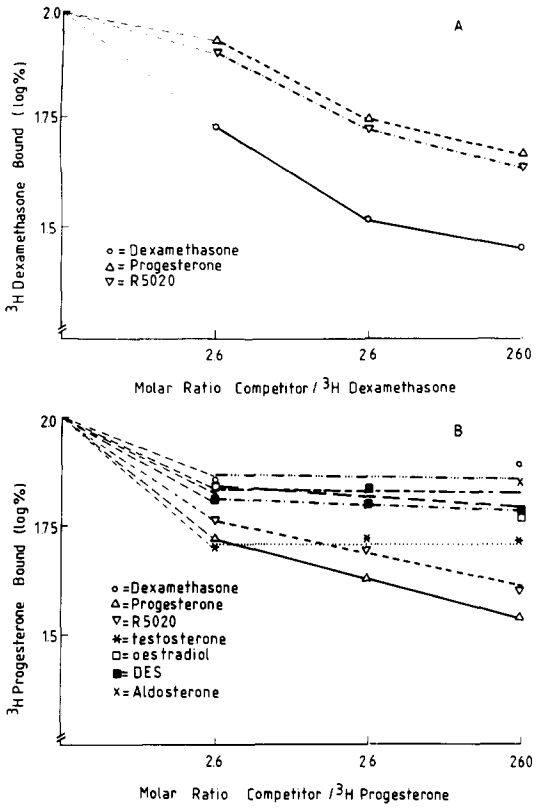

Fig. 1. Competition for the binding of [^{3}H]dexamethasone (A) or [^{3}H]progesterone (B) in intact L-cells, SL cells at concns of 1×10^7–3×10^7 cells/ml were incubated at 37° for 40 min in culture medium containing 38 nM of the indicated radioactively labelled steroid and three different excesses of each of the unlabelled competitors. Hormone binding was determined as described in Materials and methods. 100% represents the amount of bound radioactive steroids without any competitor. Each point is the average of three independent experiments. S.D. are less than 10% of the values shown.

solvents. During preincubation progesterone both saturated all of its specific binding sites and also occupied its heterospecific binding sites. These altogether resulted in a decrease to 17% of the subsequent specific dexamethasone binding, as compared to that without presaturation. On the other hand, the preincubation with dexamethasone again did not influence significantly the subsequent specific progesterone binding.

To exclude the possibility of the binding to non-receptor proteins, R5020 and cortisol were used. The former, a synthetic progestin [12], metabolically stable, does not bind to specific plasma proteins [12, 13] and is used to detect and assay progestin receptors. In all the competition assays R5020 behaved similarly to progesterone (Fig. 1), supporting the specific nature of the binding. A similar conclusion can be drawn from the fact that the pretreatment of the cells with excess cortisol (Table 1) does not influence the subsequent specific [^{3}H]progesterone binding, indicating that progesterone binding to CBG is negligible in this assay.

To further characterize the progesterone-binding site we used standard quantitative cell-free techniques. The binding of increasing concentration of [^{3}H]progesterone to receptor in cell-free extracts was plotted according to Scatchard [8]. Results of a typical experiment are shown in Fig. 2. Data were best fitted by a straight line of negative slope indicating the presence of a saturable, high-affinity binding site. Our failure to demonstrate a second binding component can be explained by supposing that the binding affinities of the two sites in the SL cells for progesterone differ substantially and at the low concns tested progesterone binds to the unique site only [14]. When data from all the Scatchard plots (N = 5) were combined by averaging the values obtained in separate experiments, the specific binding of progesterone at 4° demonstrated a K_d of 20 ± 4.2 nM and a B_{max} of 0.18 ± 0.04 pmoles/mg protein. Association (k_a) and dissociation (k_d) rate constants for progesterone at 4° in cell-free cytosol preparations were also determined; rate constants were calculated according to Munck [15]. k_a was found to be $1.94 \pm 0.5 \times 10^6\,M^{-1}\,min^{-1}$ and k_d was $6.6 \pm 0.8 \times 10^{-2}\,min^{-1}$—equivalent to a half-life of 10.5 min. The K_d value calculated from the two rate constants is in rather good agreement with that measured at equilibrium.

The progesterone-binding site of the SL cells described

Table 1. Effect of presaturation on the subsequent binding of [^{3}H]dexamethasone and [^{3}H]progesterone (% specific binding)*

Presaturation	Competitor steroid†	[^{3}H]Dexamethasone	[^{3}H]Progesterone
	In whole cells‡		
—	Dexamethasone	100	
Progesterone	Dexamethasone	17 ± 3.3	
—	Progesterone		100
Dexamethasone	Progesterone		93 ± 2.7
Cortisol	Progesterone		97 ± 2.0
	In the nuclear fraction§		
—	Progesterone		71 ± 2.0
Dexamethasone	Progesterone		74 ± 3.5

* Results represent the means of six independent experiments ± S.D.

† Competitor steroids were used in 260-fold excess.

‡ Experiments were performed as indicated under Fig. 1, except that cells were also preincubated with 5 μM or the indicated steroid for 20 min at 37°. Specific binding was determined as described in Materials and methods.

§ The intracellular distribution of the steroid was determined according to Sibley and Tomkins [6]. For details see Materials and methods.

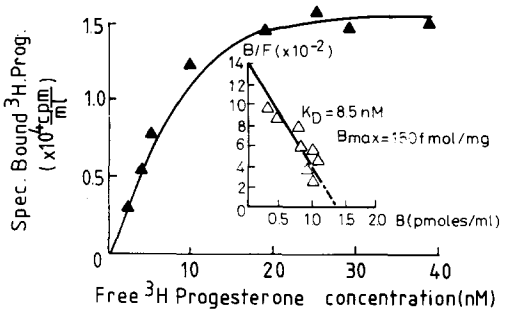

Fig. 2. Saturation and Scatchard analysis of progesterone binding in cell-free cytosol of L-cells. Aliquots of particle-free cell extracts of the SL cells were incubated at 4° for 2–4 hr with increasing concns of [^{3}H]progesterone. Binding was determined using a dextran-coated charcoal competitive binding assay as described in Materials and methods.

herein seems to be a real progesterone receptor. The binding parameters of this site are of the same order of magnitude as those reported for other tumor cell lines [16]. The competition and specificity characteristics of the binding also suggest the identity of this progesterone receptor [13, 16]. The presence of progesterone receptor in this but not in another line of the L-cells [4, 5] may reflect genetic differences between the individual lines.

The binding of a certain steroid molecule to its specific high-affinity receptor is only one of the first events in the pathway of hormone action. The steroid–receptor complex, after an activation process, is transported to the cell nucleus. Table 1 shows the proportion of specifically bound progesterone in the nuclear fraction after incubating the cells at 37° in a medium containing radioactively labelled hormone with or without prior saturation with the competitor steroid. Seventy-one per cent of the specifically bound [^{3}H]progesterone was localized in the nuclear fraction, and this ratio was not changed after dexamethasone presaturation either. The result of nuclear transfer assays shows that the formed P–R complexes are readily transported at a normal rate into the cell nuclei, which is the prerequisite of any steroid-induced response. The biological role of the progesterone receptor in the L-cells is not yet known. Our earlier results affirmed that the incorporation of [^{3}H]thymidine into the dexamethasone-sensitive A_9HT [17]—another L-cell line—was inhibited not only by dexamethasone, but—to a lesser extent—also by progesterone [2] (this test could not be performed with SL cells since they lacked thymidine kinase [18]). A_9HT cells contain specific progesterone receptors, too, and since a high proportion of the bound progesterone is localized in the cell nuclear fraction the inhibition seems to be mediated by the progesterone receptor.* Interestingly enough in the dexamethasone-resistant R^+-variants, isolated from the A_9HT cell line, the progesterone responsiveness was also diminished [2]. In cell fusion experiments we found positive complementation of the dexamethasone-resistance hybridizing SL cells with one of the resistant variants.† It would be interesting to see whether the return of the dexamethasone sensitivity in these hybrid cells is also accompanied by the return of the progesterone-responsive phenotype or the two kinds of unresponsiveness are dissociated. Experiments of this kind are in progress in our laboratory.

In summary: a high-affinity, saturable progesterone-binding site was found in transformed mouse fibroblast L-cells. By *in vivo* competition assays it was found to be different from the glucocorticoid receptor already known from these cells. Affinity characteristics and binding parameters obtained under *in vitro* conditions are similar to those reported for progesterone receptors. Receptor-bound progesterone is transported at a normal rate to the cell nuclei, which is the prerequisite of any steroid-induced biological response.

Acknowledgements—This study was supported by the Central Research Fund of the Hungarian Academy of Sciences. We wish to thank Mrs I. Bartók and Miss É. Berki for their invaluable technical assistance, and Miss Zs. Rácz and Mr B. Dusha for their help in the preparation of this manuscript.

Institute of Genetics
Biological Research Center
Hungarian Academy of Sciences
H-6701 Szeged, P.O. Box 521
Hungary

ANDRÁS GÁL
ANIKÓ VENETIANER

* A. Gál and A. Venetianer, unpublished data.

† A. Gál and A. Venetianer, *Cytogenet. Cell Genet.* (in press).

REFERENCES

1. M. E. Lippman and E. B. Thompson, *J. biol. Chem.* **249**, 2483 (1974).
2. A. Venetianer, K. Bajnóczky, A. Gál and E. B. Thompson, *Somatic Cell Genet.* **4**, 513 (1978).
3. A. Venetianer, Zs. Pintér and A. Gál, in *Advances in Physiological Science* (Eds. E. Stark, G. B. Makara, Zs. Ács and E. Endröczy), Vol. 13, p. 67. Pergamon Press, Akadémiai Kiadó, Budapest (1981).
4. I. Jung-Testas, F. Bayard and E. E. Baulieu, *Nature, Lond.* **259**, 136 (1976).
5. J. F. Hackney, N. J. Holbrook and R. J. Grasso, *J. Steroid Biochem.* **14**, 971 (1981).
6. C. H. Sibley and G. M. Tomkins, *Cell* **2**, 221 (1974).
7. J. D. Baxter and G. M. Tomkins, *Proc. natn. Acad. Sci. U.S.A.* **68**, 932 (1971).
8. G. Scatchard, *Ann. N.Y. Acad. Sci.* **51**, 660 (1949).
9. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
10. T. R. Jones and P. A. Bell, *Biochem. J.* **188**, 237 (1980).
11. F. Svec, J. Yeakley and R. Harrison, III, *Endocrinology* **107**, 566 (1980).
12. D. Philibert and J. P. Raynaud, *Steroids* **22**, 89 (1973).
13. M. R. Walters and J. H. Clark, *J. Steroid Biochem.* **8**, 1137 (1977).
14. W. B. Panko, C. S. Watson and J. H. Clark, *J. Steroid Biochem.* **14**, 1311 (1981).
15. A. Munck, in *Receptors and Mechanism of Action of Steroid Hormones* (Ed. J. R. Pasqualini), p. 1. Marcel Dekker, New York (1976).
16. M. Lippman, G. Bolan and K. Huff, *Cancer Res.* **36**, 4602 (1976).
17. F. Wiener, G. Klein and H. Harris, *J. Cell Sci.* **12**, 253 (1973).
18. J. W. Littlefield, *Nature, Lond.* **203**, 1142 (1964).