Vol. 164, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 16, 1989 Pages 480-487

ISOLATION AND CHARACTERIZATION OF A MOUSE L CELL VARIANT DEFICIENT IN GLUCOCORTICOID RECEPTORS

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Received August 23, 1989

The growth of mouse L cell fibroblasts is inhibited by glucocorticoids, and we have selected spontaneous glucocorticoid-resistant L cells in culture. One cloned variant exhibits a stable phenotype in the absence of selective conditions. This variant contains no specific glucocorticoid-binding capacity, no immunoreactive glucocorticoid receptor protein, and no detectable glucocorticoid receptor messenger RNA. A glucocorticoid-dependent reporter gene requires exogenous glucocorticoid receptor cDNA and steroid in order to be expressed in this variant. Genomic DNA analysis of the variant cell line indicates that there has been no gross alteration in receptor gene structure. These results suggest that the variant may be deficient in transcription of the glucocorticoid receptor gene.

In order to exert their effects on gene expression, glucocorticoids must first bind to their specific intracellular receptors. Genetic studies of glucocorticoid receptor (**GR**) mutants have greatly contributed to our understanding of the nature of the receptor (reviewed in Refs. 1-4). In cells of lymphoid origin, the response to glucocorticoids is often apoptosis, or programmed cell death (5, 6). Variant lymphoid cells selected for resistance to the cytolytic effect of glucocorticoids exhibit four distinct phenotypes: r^- (receptor-deficient) variants contain GR with little or no specific glucocorticoid-binding capacity (7, 8); nt^i (nuclear transfer-increased) variants contain steroid-binding GR with increased affinity for nonspecific DNA (9); nt^- (nuclear transfer-deficient) variants contain steroid-binding GR with reduced affinity for DNA (9, 10); and act^l (activation-labile) variants contain receptors with temperature-sensitive steroid-binding (11). The molecular basis for the nt^- phenotype in mouse S49 lymphoma variants has been shown to be an amino acid substitution in the DNA-binding domain of the GR protein (12). The nt^- phenotype is due to synthesis of truncated GR protein (13). For almost all r^- variants of mouse S49, mouse WEHI7, and rat

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Abbreviations and trivial names - GR, glucocorticoid receptor(s); CAT, chloramphenicol acetyltransferase; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; triamcinolone acetonide, 9α-fluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione 16,17-acetonide; dexamethasone, 9α-fluoro-16α-methyl-11β,17α,21-trihydroxypregna-1,4-diene-3,20-dione; mesylate, 21-methanesulfonate; SDS, sodium dodecyl sulfate.

0006-291X/89 $1.50

hepatoma cells, the level of receptor protein and mRNA is reduced but not absent (14, 15), consistent with the proposal that there is a threshold level of active GR below which hormone responses will not occur (16, 17).

In contrast to lymphoid cells, the response of murine L 929 fibroblasts to glucocorticoids is growth inhibition (18). Hackney *et al.* (19) isolated glucocorticoid-resistant L cells that contained 10-15% of the parent level of specific binding capacity. This subline was subsequently cloned and shown to contain a reduced level of receptors with normal affinity for glucocorticoids (20). The GR in L cells has been shown to be a 100-kDa phosphoprotein (21, 22), and there is evidence to suggest that one function of receptor phosphate is to maintain the high affinity ligand binding conformation of the protein (23). In order to isolate GR mutants that might be deficient in ligand binding as a consequence of an altered GR phosphorylation site, we have selected spontaneous glucocorticoid-resistant L cell variants in culture. In the present report, we present evidence to show that one of these variants is a novel, unambiguous r^- that lacks detectable GR protein or GR mRNA.

MATERIALS AND METHODS

Materials - [6,7-^{3}H]Triamcinolone acetonide (42.5 Ci/mmole), [6,7-^{3}H]dexamethasone mesylate (48.9 Ci/mmole), [^{125}I]goat anti-mouse IgG (5.9 μCi/μg), [α-^{32}P]deoxycytidine 5'-triphosphate (3000 Ci/mmole), [dichloroacetyl-1,2-^{14}C]chloramphenicol (60 mCi/mmole), and EN^{3}HANCE were from New England Nuclear. Restriction endonucleases and the random primer DNA labeling kit were from BRL Life Technologies. Protein A-Sepharose, purified chloramphenicol acetyltransferase (CAT), dexamethasone, diethylaminoethyl-dextran, and routine chemicals were from Sigma Chemical Co. The anti-receptor monoclonal antibody BuGR2 (24) was provided by Drs. W. J. Hendry and R. W. Harrison. The plasmid pSV2Wrec contains the mouse GR cDNA and the plasmid pMMTV-CAT contains the bacterial CAT gene under control of the glucocorticoid-dependent mouse mammary tumor virus promoter (12). These constructions were obtained from Drs. M. Danielsen, J. P. Northrop, and G. M. Ringold.

Cell culture, cytosol preparation, and steroid binding assays - Mouse L 929 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum (Hyclone). For variant selection, cells were passaged in the continuous presence of 1 μM dexamethasone for 13 months, followed by 1 μM triamcinolone acetonide for an additional 5 months. Glucocorticoid-resistant variants were cloned from single cells in medium containing steroid and subcloned in steroid-free medium. Cytosol containing 10 mM sodium molybdate was prepared from cells grown in steroid-free medium as previously described (21) and assayed for specific glucocorticoid-binding capacity with [^{3}H]triamcinolone acetonide (23). For covalent labeling experiments, cytosol was incubated with 100 nM [^{3}H]dexamethasone mesylate plus vehicle or 50 μM radioinert dexamethasone for 4 h at 0-4° C before analysis.

Immunoadsorption, gel electrophoresis, and immunoblotting - Aliquots of cytosol were mixed with an equal volume of TEG buffer (10 mM TES, 50 mM NaCl, 10% glycerol, 4 mM EDTA, pH 7.6 at 4° C). Monoclonal antibody or an equal concentration of nonimmune mouse IgG was added to the mixture at 5% of the final volume and the samples were incubated on ice for 12-16 h. Protein A-Sepharose (50 μl) equilibrated in TEG buffer was added, and the samples were mixed by continuous rotation at 4° C for 2 h. The matrix was pelleted by centrifugation and washed 5 times by suspension in 1 ml of TEG buffer containing 0.4 M NaCl and 0.2% Triton X-100, followed by 3 washes with TEG buffer. The pellets were boiled in SDS sample buffer and the eluted proteins were resolved by SDS-

polyacrylamide gel electrophoresis on 7% polyacrylamide gels according to the method of Laemmli (25). Gels were cooled to 5° C during electrophoresis. Molecular weight standards were: myosin (205-kDa), β-galactosidase (116-kDa), phosphorylase-*b* (97.4-kDa), bovine serum albumin (66-kDa), ovalbumin (45-kDa), and carbonic anhydrase (29-kDa). For detection of [^{3}H]dexamethasone mesylate-labeled proteins, gels were impregnated with EN^{3}HANCE and fluorographed. For immunoblotting experiments, resolved proteins were transferred to Immobilon P membranes (Millipore) and probed with 1% BuGR2 anti-receptor antibody as previously described (22). The immunoreactive proteins were detected by autoradiography after incubation with [^{125}I]goat anti-mouse IgG (26).

RNA and DNA blot analysis - Total cellular RNA from cultured cells was prepared as described by Wood *et al.* (27). Samples containing 15 μg of formamide-denatured RNA were separated by electrophoresis in a formaldehyde-agarose gel and transferred to a nylon membrane (HyBond N, Amersham) as described by Northrop *et al.* (15). Prehybridization and hybridization conditions were essentially as described (15) except that hybridization solution contained 10% dextran sulfate and incubations were done at 42° C. Membranes were washed at room temperature for 3 X 15 min in 2X SSPE, 0.1% SDS and for 2 X 15 min in 0.2X SSPE, 0.1% SDS before autoradiography. The 1 kb *Hin*dIII fragment of mouse GR cDNA (12) was radiolabeled with [α-^{32}P]dCTP by the random priming method (28) and used as the probe for both GR mRNA and GR genomic DNA sequences. A 770 bp fragment of the chicken actin gene (Oncor) was labeled by the same method and used to detect actin mRNA on RNA blots. For rehybridization to the actin probe, the membrane was first stripped of radioactivity by washing with 0.1X SSPE, 0.1% SDS at 95° C. Genomic DNA was prepared from cultured cells (29) and digested with the indicated restriction enzyme (5 U/μg). Twenty μg of each sample were separated on agarose gels as described (15) and transferred to charge-modified nylon membranes (HyBond N-plus, Amersham) in 0.4 M NaOH. Prehybridization and hybridization conditions were identical to RNA blots.

Cell transfection and CAT assays - Cells growing in 150 cm^2 flasks were transfected with plasmid DNA using the diethylaminoethyl-dextran method (30) as modified by Selden *et al.* (31). Cells were washed with Tris-buffered saline (25 mM Tris, 137 mM NaCl, 5.1 mM KCl, 1.4 mM Na_2HPO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.5 at 25° C) and incubated for 4.5 h in serum-free medium containing 200 μg/ml diethylaminoethyl-dextran and plasmid DNA. Each flask received 12 μg of pSV2Wrec and/or 20 μg of pMMTV-CAT. After aspirating this solution, cells were incubated for 90 sec in a solution of 10% dimethyl sulfoxide in phosphate-buffered saline (4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.35 at 25°) and washed twice with phosphate-buffered saline. Fresh medium containing 10% calf serum was added and transfected cells were incubated for 3 days and harvested. For induction of CAT, dexamethasone (1 μM) was added 16 h before harvest. Cell extracts containing 5 mM EDTA were prepared as suggested by Crabb and Dixon (32) and assayed for CAT activity as described by Gorman *et al.* (33). The acetylated products were separated by thin-layer chromatography and visualized by autoradiography (33).

RESULTS

As L cells are inhibited but not killed by glucocorticoids, it was necessary to propagate these cells for a prolonged period in the presence of steroid in order to select a spontaneous glucocorticoid-resistant variant population. Following selection in dexamethasone, the steroid was changed to triamcinolone acetonide in order to preclude isolating membrane permeability variants resistant to dexamethasone but not to triamcinolone acetonide (34). One steroid-resistant cloned variant, designated E8.2, was chosen for further study. Cytosol from this variant exhibited no detectable specific glucocorticoid-binding capacity, even after continuous culture for six months in steroid-free

medium. This suggests that the mutation is stable and does not revert at an appreciable frequency.

In order to detect receptors in the variant that might have reduced affinity for reversible association with glucocorticoids, cytosol was incubated with the covalent GR affinity ligand [3H]dexamethasone mesylate (35). Figure 1 shows that parent L cell cytosol contains a 100-kDa protein which is covalently labeled by the site-specific ligand in a displaceable manner. This protein has previously been shown to be the GR (22). E8.2 cell cytosol does not contain detectable proteins of any size that are specifically labeled by [^{3}H]dexamethasone mesylate, even after concentration by immunoadsorption to protein A-Sepharose with the anti-receptor antibody.

E8.2 cells were also examined for the presence of immunoreactive GR protein. Figure 2 shows the results of an immunoblot analysis of parent L cell and variant E8.2 cell proteins after immunoadsorption. There was no detectable immunoreactive protein in E8.2 cells using BuGR2 (Figure 2) or other anti-receptor antibodies (data not shown).

As the variant cells contain no detectable receptor protein of any size, we wanted to determine if this variant was defective in the synthesis of GR mRNA. Total cellular RNA was isolated from both cell lines and fractionated on denaturing agarose gels. Figure 3 shows the results of a representative RNA blot. Parent L cells contain a single GR mRNA of 7 kb, similar to other murine cell lines (19, 36). Variant E8.2 cells do not contain any detectable GR mRNA of any size, even after extensive exposure of the blots.

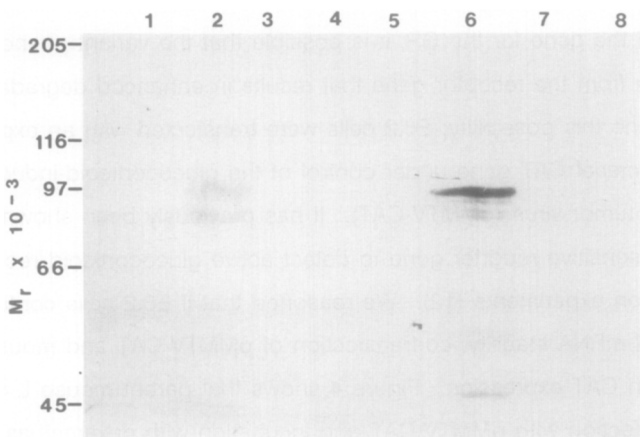

Figure 1. [^{3}H]Dexamethasone mesylate labeling. Cytosol from L cells (lanes 1,2,5,6) or E8.2 cells (lanes 3,4,7,8) was labeled with [^{3}H]dexamethasone mesylate in the presence of vehicle (lanes 2,4,6,8) or unlabeled dexamethasone (lanes 1,3,5,7). For direct analysis, aliquots (150 μl) were heated in SDS sample buffer (lanes 1-4). For concentration of GR proteins, aliquots (700 μl) were immunoadsorbed with anti-receptor antibody before preparing gel samples (lanes 5-8). [^{3}H]dexamethasone mesylate-labeled proteins were visualized by fluorography.

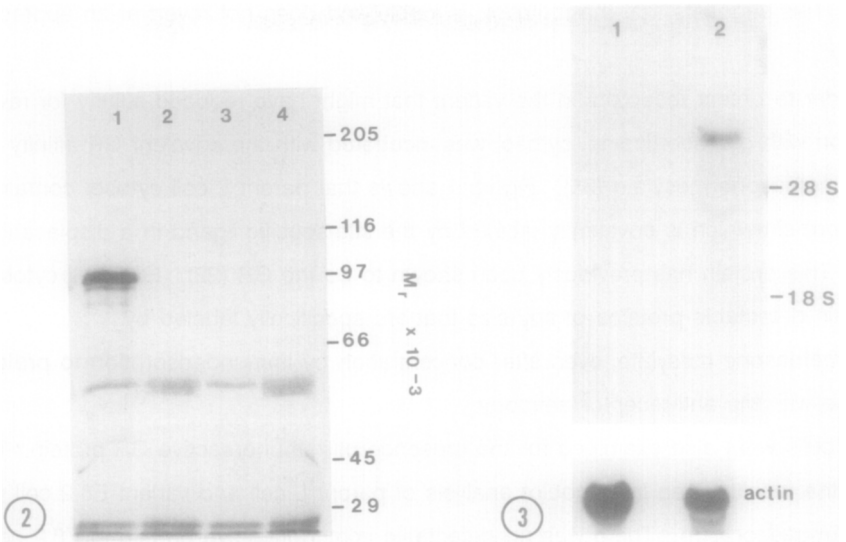

Figure 2. Immunoblot of cytosol proteins. Aliquots (0.9 ml) of L cell cytosol (lanes 1-2) or E8.2 cell cytosol (lanes 3-4) were immunoadsorbed with BuGR2 anti-receptor antibody (lanes 1, 3) or nonimmune mouse IgG (lanes 2, 4), resolved by SDS gel electrophoresis, and immunoblotted with BuGR2 antibody. Immunoreactive proteins were visualized by autoradiography after incubation with [^{125}I]anti-mouse IgG. The common bands at 55-kDa and 25-kDa correspond to the heavy and light chains of IgG.

Figure 3. RNA blot analysis. Total RNA from E8.2 cells (lane 1) and L cells (lane 2) was probed for GR mRNA sequences using a ^{32}P-labeled mouse GR cDNA fragment which spans 45% of the coding sequences (top). After autoradiography, the blot was stripped of bound radioactivity and probed for actin mRNA sequences (bottom). The migration of the 28 **S** (4.8 kb) and 18 **S** (1.9 kb) ribosomal RNA bands is shown on the right.

Although these results suggest that the lack of detectable GR in the variant cells is due to an alteration in the gene for the GR, it is possible that the variant phenotype is due to a mutation separate from the receptor gene that results in enhanced degradation of GR mRNA. To examine this possibility, E8.2 cells were transfected with an expression plasmid containing the bacterial CAT gene under control of the glucocorticoid-inducible promoter of mouse mammary tumor virus (pMMTV-CAT). It has previously been shown that this construction is a sensitive reporter gene to detect active glucocorticoid receptors in transient expression experiments (12). We reasoned that if E8.2 cells contain a factor that interfered with GR mRNA stability, co-transfection of pMMTV-CAT and mouse GR cDNA would not result in CAT expression. Figure 4 shows that parent mouse L cells express CAT activity after transfection with pMMTV-CAT and incubation with dexamethasone to occupy GR and activate gene transcription. Conversely, E8.2 cells do not express CAT under these conditions. However, when E8.2 cells are co-transfected with mouse GR cDNA (pSV2wrec), the expression of CAT is induced by dexamethasone. These results demonstrate that E8.2 cells do not contain active endogenous GR and that there is not a mutation separate from the GR gene that results in rapid degradation of GR mRNA.

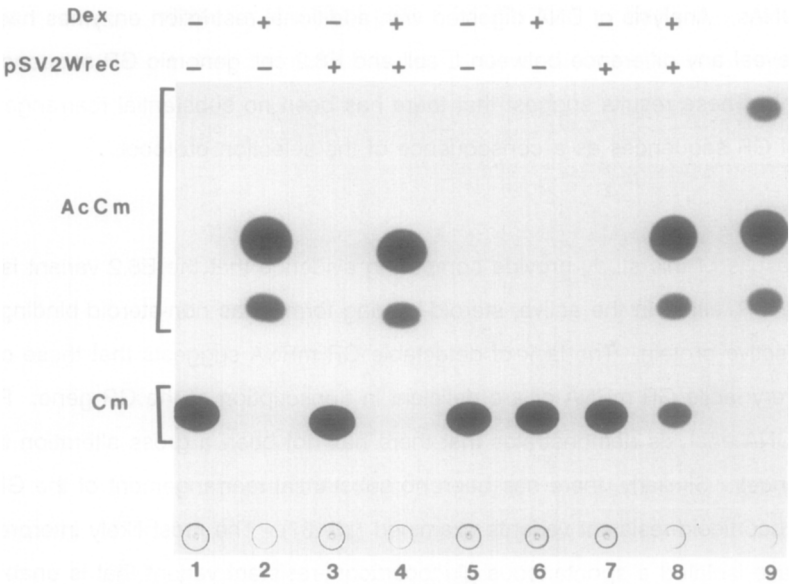

Figure 4. Expression of pMMTV-CAT in variant cells. Sets of L cells (lanes 1-4) or E8.2 cells (lanes 5-8) were transfected with pMMTV-CAT DNA, and one-half of each set was co-transfected with pSV2Wrec DNA. Dexamethasone or vehicle was added to each set of cells before assaying for CAT activity as indicated. The acetylated products (AcCm) were separated from chloramphenicol (Cm) by thin-layer chromatography and visualized by autoradiography. A control reaction with purified CAT enzyme is also shown (lane 9).

In order to detect an alteration in the variant GR gene, genomic DNA from L cells and E8.2 cells was analyzed with a GR cDNA probe and compared with mouse liver DNA. Figure 5 shows that the respective restriction enzyme fragments are comparable in size for

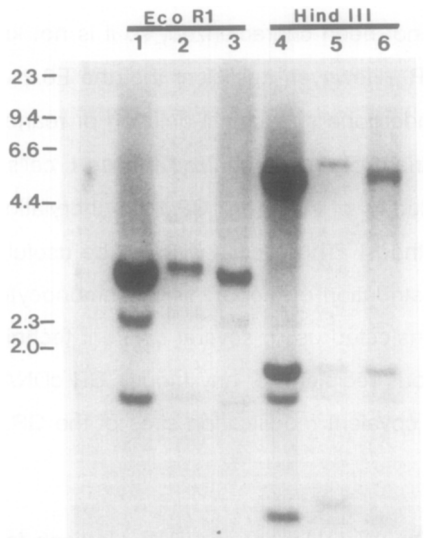

Figure 5. DNA blot analysis. Genomic DNA samples from C57/B6 mouse liver (lanes 1, 4), L cells (lanes 2, 5), and E8.2 cells (lanes 3, 6) were digested with *Eco*RI or *Hin*dIII and analyzed for mouse GR sequences using the ^{32}P-labeled mouse GR cDNA probe. Markers (in kbp) are shown on the left.

all three DNAs. Analysis of DNA digested with additional restriction enzymes has so far failed to reveal any difference between L cell and E8.2 cell genomic GR sequences (data not shown). These results suggest that there has been no substantial rearrangement or deletion of GR sequences as a consequence of the selection protocol.

DISCUSSION

The results of this study provide compelling evidence that the E8.2 variant is unable to synthesize GR, either in the active, steroid-binding form or as non-steroid-binding, immunoreactive protein. The lack of detectable GR mRNA suggests that these cells either produce very labile GR mRNA or are deficient in transcription of the GR gene. Preliminary genomic DNA analysis demonstrates that there has not been a gross alteration in genomic GR sequences. Similarly, there has been no substantial rearrangement of the GR genes in other glucocorticoid-resistant variants examined (15, 37). The most likely interpretation is that we have isolated a spontaneous glucocorticoid-resistant variant that is unable to express the GR gene, perhaps due to a promoter mutation. Experiments are presently underway to determine the nature of the mutation.

Cells that are unable to synthesize GR mRNA are unambiguous r^- variants, and only a few have previously been described. Murine P1798 lymphosarcoma cells are hemizygous for the GR, and a spontaneous variant (S20d) has been isolated that is unable to express GR mRNA as a consequence of inactivation of the functional GR allele (36). The EDR3 variant isolated from mutagenized Fu5 rat hepatoma cells lacks detectable GR mRNA (38), although genomic GR sequences were not examined. In rat hepatoma cells, the r^- phenotype is often a result of the loss of genomic GR sequences (15). The organization of the GR gene in L cells has not been characterized, so it is not known if these cells are diploid or haploid for the GR. However, it is clear that the E8.2 variant contains GR sequences that have not undergone substantial deletion or rearrangement.

The unambiguous r^- variant of unmutagenized mouse L cells has been used in this laboratory as a control in studies of intact cell GR phosphorylation and to standardize quantitative immunoblot methods. These cells will also be useful controls in experiments to determine the subcellular distribution of the GR using immunocytochemical methods. Mouse L cells are easily transfected using several different techniques, and the E8.2 variant can be used as a homologous recipient for any mouse GR cDNA construction to examine the functional domains and covalent modification sites of the GR.

ACKNOWLEDGMENTS

The authors wish to thank Drs. W.J. Hendry and R.W. Harrison for generously providing the BuGR2 antibody, Drs. M. Danielsen, J.P. Northrop, and G.M. Ringold for kindly providing both helpful advice and the pMMTV-CAT and pSV2Wrec plasmids, and Dr. F. G. Berger for mouse liver DNA. The technical assistance of Thomas R. Polte is appreciated. This work was supported by National Institutes of Health grant DK36905, American Cancer Society

grant IN-748387, and a research grant from the Pharmaceutical Manufacturers Association Foundation.

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