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Western blot analysis of glucocorticoid receptor phosphoisoforms by one- and two-dimensional electrophoretic assays

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Abstract: The glucocorticoid receptor (GR) protein is a cytosolic ligand-dependent transcription factor with numerous functions regulated by post-translational modifications, including phosphorylation/dephosphorylation. Among the functions most extensively affected by GR phosphorylation are the modulation of its transcriptional activity, alterations in its interaction pattern with cofactors, nuclear translocation and selective gene transactivation. Intensive analysis of the intracellular distribution of GR phosphoisoforms and their interaction with proteins of other cellular signalling networks required the use of [γ^{32}P]ATP as a phosphate donor, and special laboratory protection measures to avoid external irradiation and contamination. In the present study, simple and easy-to-use non-radioactive protein mobility shift assays (NMS assays) were developed using one- and/or two-dimensional gel electrophoresis based on differences in the pI and molecular mass of GR phosphoisoforms. The GR isoforms were immunodetected with specific monoclonal or polyclonal anti-GR antibodies by Western blot in three diverse systems, namely yeast BJ2168 cells expressing wild-type rat GR, rat hepatoma GRH2 cells grown in culture and brain tissue from Wistar rat experimental animals. The results obtained using the NMS assay were similar to previous results obtained with the [γ^{32}P] ATP standard assay.

Keywords: glucocorticoid receptor; phosphoisoforms; electrophoretic assay.

INTRODUCTION

The glucocorticoid receptor (GR) protein is a ligand-dependent transcriptional factor which, in the absence of glucocorticoid hormones (GCs), is located primarily in the cytoplasm of the target cell in the form of a hetero-oligomeric

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complex with heat shock proteins (HSPs).[1,2] After binding to GCs, the GR undergoes conformational changes, dissociates from the HSPs, homodimerizes and translocates into the nucleus where it interacts directly with its specific DNA sequences in the promoter region of the target genes.[1] In addition, the activated GR may interact with other transcription factors, such as the nuclear factor-κB, the activator protein-1 and many others, *via* protein–protein interactions, thus influencing indirectly the activity of these transcription factors on their target genes.[3,4]

Phosphorylation is a very important post-translational modification of GR, regulated by cellular enzymes kinases and phosphatases, influencing and modulating virtually all of the GR functions.[5,6] The *N*-terminal domain of rat GR contains phosphorylation sites at the amino acids serine 224/232 and serine 246, activated by cyclin-dependent kinases (CDKs) and c-Jun-*N*-terminal kinase (JNK), respectively.[7,8] GR phosphorylation by these kinases affects its transcriptional activity, protein stability and nucleo-cytoplasmic shuttling, thus modulating GR-mediated gene expression.[5,9,10] For an understanding of the physiological functions of GR, it is of crucial importance to have simple and accurate method(s) for analysis of the phosphoisoforms of GR. Such methods would enable studies of intracellular distribution of GR phosphoisoforms and their interaction with proteins of other cellular signalling networks. Most of the recently used methods employed radioactively labelled phosphate donor [γ-^{32}P]ATP to follow GR phosphorylation *in vivo* or *in vitro*.[7–10] Although powerful, these techniques required special laboratory protection protocols due to possible external irradiation and contamination by the high levels of [γ-^{32}P]ATP radioactivity.[7,10]

In the present study, simple and easy-to-use non-radioactive protein mobility shift assays (NMS assays) were developed for the analysis of GR phosphoisoforms based on the differential mobility of GR phosphoisoforms separated by the one- or two-dimensional gel electrophoresis. The hyperphosphorylated GR isoform was detected by highly specific monoclonal anti-GR antibodies or epitope specific polyclonal antibodies by Western blot immunodetection assay. The NMS assays were first established *in vivo* with yeast *Saccharomyces cerevisiae* BJ2168 strain expressing wild-type rat GR and thereafter successfully applied to test *in vivo* phosphorylation of GR in rat hepatoma GRH2 cells grown in culture or brain tissue from Wistar rat experimental animals. The obtained results suggest that NMS assays can be used across very different biological systems from yeast cell to *in vivo* animal models with high sensitivity and reproducibility and provide an alternative way to study this subject and avoid using [γ-^{32}P]ATP.[7,10]

EXPERIMENTAL

Baker's yeast Saccharomyces cerevisiae *strains and plasmids*

The triple protease deficient yeast strain BJ2168 (a, pep 4-3, prc 1-417, prb 5-1122, ura 3-52, trp 1, leu 2)[11] served as the parent for derivative strains containing various 2μ-origin--based expression and reporter vectors. Transformations were performed by the lithium ace-

tate procedure.[12] The yeast expression plasmid pG-N795[13,14] contained rat GR cDNA driven by the yeast glyceraldehyde-3-phosphate dehydrogenase promoter, residing on a 2μ vector (10–40 copies per cell) bearing the TRP1 selectable marker. The 2μ vector carries the URA3 selectable marker, a bacterial origin of replication and the bacterial ampicillin resistance gene.[14] Yeast cultures were propagated at 30 °C in minimal yeast medium supplemented with a standard mixture of amino acids and 2 % glucose.[15]

Mammalian cells culture

The mammalian cell line GRH2, a derivative of transfected hepatoma HTC cells, expressing a high level GR was used in the study.[16] Cells were grown in 90 % Dulbecco's modified Eagle's medium H-16, supplemented with 10 % heat-inactivated FCS, 100 IU/mL penicillin and streptomycin and 2 mM L-glutamine (all obtained from Sigma-Aldrich, Taufkirchen, Germany) in 95 % humidity atmosphere of 95 % air and 5 % CO_2 at 37 °C.

Hormone treatment and preparation of yeast and GRH2 cell extracts

Yeast and GRH2 cells were treated *in vivo* with 10 μM deoxycorticosterone (DOC, 3300 ng/mL medium) or 10 μM dexamethasone (Dex, 3900 ng/mL medium), respectively. The yeast cell extract was prepared (1:2 mass/volume) at 4 °C by glass bead lysis in 45 mM Hepes pH 7.4, containing 10 % glycerol, 1 mM Na_2EDTA, 400 mM NaCl, 1 mM DTT and 0.5 % Nonidet P40 followed by centrifugation at 13,000 rpm in a Beckman JA centrifuge. When indicated, the supernatant was treated for 30 min at 25 °C with 20 IU/mL of calf intestine phosphatase (Boehringer Mannheim). The GRH2 cell extract was prepared in the same manner as the yeast extract except that the glass beads were omitted.

Preparation of cell extracts from brain cortex of experimental animals

Wistar males (2 months old, body mass 250 g) were exposed to acute stress (immobilization),[17] sacrificed by decapitation and the prefrontal brain cortex (PFC) was weighed and homogenized (1:2 mass/volume) at 4 °C in 20 mM Tris HCl buffer pH 7.2, containing 10 % glycerol, 1 mM Na_2EDTA, 1 mM Na_2EGTA, 50 mM NaCl, 2 mM DTT and protease inhibitors.[17] The homogenate was centrifuged at 38000 rpm for 60 min at 4 °C and the resulting supernatant was used as cell cytosol.

The protein concentration in the yeast and mammalian cell extracts or brain cell cytosol was determined by the method of Lowry.[18]

Separation and analysis of samples by one-dimensional gel electrophoresis (1-DE) or two-dimensional gel electrophoresis (2-DE)

For analysis by 1-DE, samples (60 μg of protein) were mixed (1:1) in denaturing buffer according to Laemmli[19] and separated by molar mass (M_m) in 7.5 % slab polyacrylamide gels for 45 min at 100 V under denaturing conditions. Standard mixture of M_m marker proteins (Sigma SDS6H2), containing: equine myosine (220000 g/mol), *Escherichia coli* β-galactosidase (116000 g/mol), rabbit muscle phosphorylase B (97400 g/mol), bovine albumin (66000 g/mol) and egg white ovalbumin (45000 g/mol), was used for 1-D gel calibration.

In 2-DE, samples (100 μg of protein) were first mixed with isofocusing buffer (9 M urea, 2 % Triton X-100, 5 % β-mercaptoethanol, 2 % of ampholines (4 volumes of ampholine pH 5–7 plus 1 volume of ampholine pH 3–10) and 0.1 % Bromphenol Blue), and separated according to isoelectric point (pI) in prefocused 4 % disk polyacrylamide gels. The gels were prefocused for 20 min at 200 V, then 20 min at 300 V and 20 min at 400 V before sample isofocusing was run for 10 min at 500 V and then 3.5 h at 750 V. For pI calibration, the following pI markers were run in parallel gel disks: soybean trypsin inhibitor (TI, pI = 4.6),

bovine carbonyl anhydrase (CA, pI = 5.9) and equine myoglobin (Myo, pI = 7.0). The sample disks were taken out of the glass tubes, incubated for 60 min in denaturing buffer according to Laemmli[19] and layered on top of 7.5 % slab polyacrylamide denaturing gels (the same those used in 1-DE). The electrophoretic separation according to molar mass (M_m) was performed for 90 min at 120 V.

Western blot immunodetection of GR protein

Proteins separated by 1-DE or 2-DE were electrophoretically transferred to PVDF membranes and blocked for 1 h at room temperature in phosphate buffered saline (PBS) containing 5 % non-fat dry milk. Immunopositive bands were detected either with GR-specific BUGR2 (gift from Professor Keith R. Yamamoto) monoclonal antibody or with M-20 (sc-1004, Santa Cruz Biotechnology) polyclonal antibody, both prepared in PBST (PBS, 0.1 % Tween 20, 2.5 % milk). After extensive washing (3–5 times for 10 min in PBST), the immunopositive bands were visualized by secondary goat-anti mouse or goat-anti rabbit antibody linked to alkaline phosphatase (ALP)[20] or horseradish peroxidase chemiluminescences (ECL) detection system.[17]

RESULTS AND DISCUSSION

An electrophoretic mobility shift assay, also called "gel shift assay", is a common technique usually used to study protein–DNA or protein–RNA interactions by 1-D electrophoresis (1-DE). In the present report, it is shown that 1-DE and 2-DE methods can also be successfully applied for monitoring the phosphorylation or dephosphorylation of a single protein, *i.e.*, glucocorticoid receptor (GR) isolated from yeast *Saccharomyces cerevisiae* cells or mammalian cells. As may be observed in Fig. 1A (lanes 1, 3 and 5), the native unliganded GR expressed in yeast (*S. cerevisiae* strain BJ2168) resolved by 1-DE was detected by Western blot using specific anti-GR monoclonal antibodies (BUGR) as a single protein band corresponding to the position of the phosphorylase B marker at 97400 g/mol. Upon addition of the yeast GR specific hormone analogue deoxycorticosterone (10 μM, *i.e.*, 3300 ng DOC/mL medium, K_d 4 nM),[15,21] a "smeared" GR pattern appeared with a GR band corresponding to M_m of about 99000 g/mol (99kGR) (Fig. 1A, lanes 2, 4 and 6). The shift in the M_m of GR was observed in the interval of 50–100 μg of total proteins assayed per lane (Fig. 1A, ALP detection) up to 200 μg of total proteins assayed (Fig. 1B, ECL detection). Earlier studies[3,10] in *S. cerevisiae* BJ2168 mutant strains carrying the GR serine 232 site targeted by CDK for phosphorylation mutated to alanine showed that the ligand-dependent shift in the M_m of GR corresponds to its hyper-phosphorylated form.[3,10] In order to check if the 99kGR was actually a hyper-phosphorylated form of the GR, a control experiment in which BJ2168 extract was treated *in vitro* with 20 IU/mL of calf intestine phosphatase was performed (Fig. 1B). As may be observed, the ligand-dependent 99kGR was not present after the phosphatase treatment (Fig. 1B, lane 4). This fact complements and extends previous findings[10] and provides a strong indication that 99kGR band was indeed the hyper-phosphorylated isoform of GR.

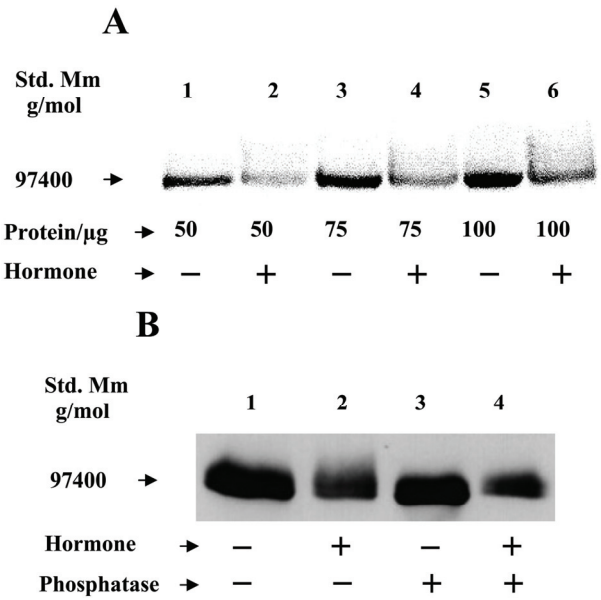

Fig. 1. Western blot analysis of yeast BJ2168 ectopically expressing GR separated by 1-DE. A) Titration of protein concentration 50, 75 or 100 µg and mobility shift: controls (lanes 1, 3, and 5), and yeast GR after hormone treatment (lanes 2, 4 and 6). B) Mobility shift sensitivity to phosphatase treatment: controls (lanes 1 and 3), yeast GR after hormone treatment (lanes 2 and 4) and after calf intestine phosphatase (lanes 3 and 4). The position of molar mass standard (Std. M_m) phosphorylase B (97400 g/mol) is indicated by an arrow.

When untreated or DOC treated BJ2168 extracts were resolved by 2-DE (1--isoelectrofocusing and 2-separation according to molar mass) and screened by BUGR, both pI and M_m shifts were observed (Fig. 2, left and right panels, respectively). The ligand-bound, hyper-phosphorylated and activated form of GR[22] was shifted towards both higher M_m and more acidic pI. The same 2-DE analysis was performed with GRH2 cell extracts after *in vivo* treatment with 10 µM Dex (3900 ng Dex /mL medium, K_d 4nM,[23] Fig. 3). A similar, although less pronounced, shift in the M_m and pI of GR was observed, indicating that GR phosphoisoforms may be also analysed by the 2-DE of rat hepatoma GRH2 cells in culture.

Finally, GR phosphorylation was assayed by the 1-DE shift-assay in extracts isolated from Wistar rat brain tissue. The prefrontal brain cortex extracts which contain high levels of GR protein[24] were prepared from control animals in which the natural hormone corticosterone (CORT) was at a low diurnal level, *i.e.*, 130 ng/mL plasma (Fig. 4, lane 1). The cytosol was also prepared from stressed animal brain corresponding to 630 ng of CORT/mL plasma (Fig. 4, lane 2). The 1-DE analysis followed by Western blot detection of GR by specific M-20 polyclonal antibodies clearly showed the appearance of both 97.4kGR and 99kGR hormone-dependent hyperphosphorylated isoform under high CORT conditions.

These results provide evidence that the employed method may be successfully applied to separate these phosphoisoforms *in vivo* in whole animal studies. Moreover, in recent publication in which serine 232 epitope specific anti-GR antibodies were used, the hyperphosphorylated isoform with reduced mobility (99kGR) was shown to be a CDK phosphorylated form of GR in Wistar rat brain.[17]

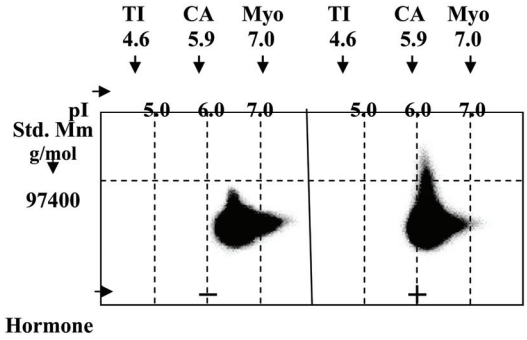

Fig. 2. Western blot analysis of yeast BJ2168 ectopically expressing GR separated by 2-DE. Left panel: the control sample; right panel: the sample treated with hormone. The positions of the pI standards: soybean trypsin inhibitor (TI, pI = 4.6), bovine carbonic anhydrase (CA, pI = 5.9) and equine myoglobin (Myo, pI = 7.0), and the position of the molar mass standard (Std. M_m) phosphorylase B (97400 g/mol) are indicated by dashed lines.

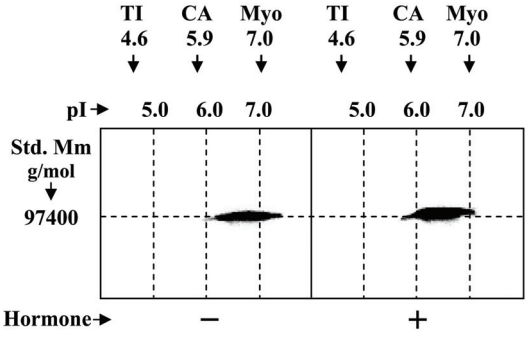

Fig. 3. Western blot analysis of the GR from rat liver GRH2 cells separated by 2-DE. Left panel: the control sample; right panel: the sample treated with hormone (10 μM Dex). The position of the pI standards: soybean trypsin inhibitor (TI, pI = 4.6), bovine carbonic anhydrase (CA, pI = 5.9) and equine myoglobin (Myo, pI = 7.0), and the position of the molar mass standard (Std. M_m) phosphorylase B (97400 g/mol) are indicated by dashed lines.

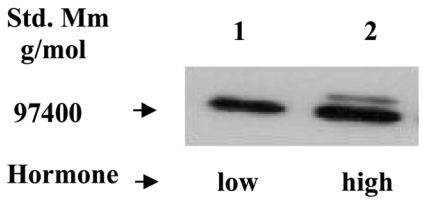

Fig. 4. Western blot analysis by specific M-20 antibodies of the GR from the prefrontal cortex of Wistar rat brain separated by 1-DE: control sample with low hormone level (130 ng/mL CORT) (Lane 1) and a stressed animal with a high hormone level (630 ng/mL CORT), (Lane 2). The position of the molar mass standard (Std. M_m) phosphorylase B (97400 g/mol) is indicated by an arrow.

CONCLUSIONS

The non-radioactive electrophoretic protein mobility shift assays (NMS assays) used in the present study are equivalent to a standard radioactivity based assay using [γ-^{32}P]ATP as a phosphate donor. Instead of the radioactivity detection

method, the NMS assay is based on Western blot immunodetection of GR phosphoisoform separated in one- and/or two dimensional gels according to differences in pI and molar mass. The GR forms are easily detected in all the studied biological systems and GR undergoes hormone dependent phosphorylation in yeast as well as in mammals, suggesting the importance and conservation of this process. In addition, these GR isoforms can be detected and quantified with high sensitivity and reproducibility, thus enabling the avoidance of the use of the [γ-^{32}P]ATP assay; and the method does not require special laboratory protection measures. The NMS assay may be used to analyse GR from the diverse biological systems: yeast cells, mammalian cells (rat hepatoma GRH2 culture) and brain tissue from experimental animals.

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ИЗВОД

«WESTERN BLOT» АНАЛИЗА ФОСФОИЗОФОРМИ ГЛУКОКОРТИКОИДНОГ РЕЦЕПТОРА ПОМОЋУ ЈЕДНОДИМЕНЗИОНАЛНЕ И ДВОДИМЕНЗИОНАЛНЕ ЕЛЕКТРОФОРЕЗЕ

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Глукокортикоидни рецептор (ГР) је цитосолни лиганд-зависни транскрипциони фактор чије бројне функције су регулисане пост-транслационим модификацијама: фосфорилацијом и дефосфорилацијом. Међу функцијама ГР које су најосетљивије на фосфорилацију су: модулација његове транскрипционе активности, промене у начину интеракције са кофакторима, транслокација ГР у једро и селективна трансактивација гена. У литератури су до сада описане бројне анализе унутарћелијске дистрибуције ГР и његове интеракције са протеинима других сигналних путева, које су засноване на [γ-^{32}P]ATP као донору фосфатне групе. За овакве анализе потребне су специјалне лабораторијске мере заштите од зрачења и радиоактивне контаминације. У приказаној студији развили смо једноставан и лак нерадиоактивни тест («NMS») за анализу ГР фосфоизоформи који се заснива на промени у њиховој покретљивости у једнодимензионалној или дводимензионалној електрофорези, као последици разлика у pI и молекулској маси. Фосфоизоформе ГР су анализиране у три различита биолошка система: у квасцу BJ2186 са дивљим типом («wild-type») ГР, у ћелијама хепатома пацова GRH2 гајеним у култури и у можданом ткиву експерименталних Wistar пацова. Анализа фосфоизоформи ГР је рађена имунолошком методом помоћу високо-специфичних моноклонских или поликлонских антитела коришћених у «Western blot» тесту. Резултати добијени «NMS» тестом били су слични нашим претходним резултатима у којима је коришћен стандардни [γ-^{32}P]ATP тест.

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