Insulin potentiates the transactivation potency of the glucocorticoid receptor

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Abstract A single copy of a glucocorticoid-responsive element (GRE) is sufficient in mediating the combinatorial response of a promoter to both glucocorticoids and insulin in HepG2 cells. This requires the presence of active glucocorticoid receptor (GR) since the response is significantly inhibited by the anti-glucocorticoid RU30406. The N′- and C′-terminal parts of the GR protein are not involved in mediating the response. Insulin had no effect on GR binding to GRE but it affected both the level and the phosphorylation state of nuclear-bound GR. Thus, insulin alters the GR transactivation potency while, concomitantly, modifies the molecule at the posttranslational level.

Key words: Glucocorticoid receptor; Insulin; Phosphorylation; HepG2 cell

1. Introduction

Glucocorticoid hormones exert their action by binding to an intracellular glucocorticoid receptor (GR) which undergoes conformational changes, rapidly translocates to the nucleus and by binding to target DNA sequences (termed GREs), modulates gene transcription (for review see [1]). Cloning of the cDNA coding for the GR revealed protein domains responsible for its function including steroid binding, DNA binding, as well as transcriptional transactivation [2,3]. These domains are also thought to function by interacting directly or indirectly with basal transcription machinery [4]. Some reports suggested that signalling cross-talk confers interaction of GR with other signal-dependent transcription factors resulting in positive or negative synergism on gene transcription [5]. Synergistic transactivation has been observed between GR and other factors. That could occur either by contacting of the different activators in separate targets [6] or by interaction of a GR complex with the other factors via a distinct domain [7].

Steroid hormone receptors have been shown to exist as phosphoproteins in intact cells but the exact role of phosphorylation in their action has not been defined as yet. All steroid receptors exhibit ligand-dependent phosphorylation mainly on Ser/Thr residues, which is complicated and proceeds at multiple steps. Rabbit uterine progesterone receptor undergoes two phosphorylation reactions, a cytosolic and a nuclear which is hormone-regulated [8]. Mouse uterine oestrogen receptor has also been shown to undergo nuclear phosphorylation in vivo [9].

Glucocorticoid receptor (GR) is phosphorylated in vitro in rat liver cytosol by cAMP-dependent protein kinase and in vivo in the liver of adrenalectomized rat [10]. GR is also phosphorylated in intact mouse fibroblasts (L cells) [11]. In rat liver cytosol, GR appeared as a major phosphoprotein of M_r 92,000 Da, while in L cells had an apparent M_r of 90,000 Da. Using protease digestion and chemical cleavage reagents it was demonstrated that GR is phosphorylated mainly in τ1: a potent transactivation domain lying in the NH_2-terminal region of the molecule, on serine and threonine residues [12]. The DNA-binding domain of GR is also phosphorylated but phosphorylation of the hormone-binding domain has not been reported [13–15]. Modulation of GR function by the cAMP-dependent protein kinase (PKA) was demonstrated in F9 embryonal carcinoma cells. PKA enhanced the hormone-dependent transactivation of GR in these cells at least partly by increasing the DNA-binding activity of the receptor while its affinity for the steroid was unaffected [16].

In a previous report [17] we demonstrated that insulin signalling increases the glucocorticoid inducibility on the MMTV-LTR promoter by 2-fold. We presented evidence that the synergistic effect was GR-dependent and sequence specific and that it affected the initiation rate of RNA synthesis. Here we analyze the insulin effect on the functional properties of the GR as well as on its phosphorylation pattern. Our results clearly demonstrate that the increase of the GR transactivation by the combinatorial action of both hormones complies with modulation of GR phosphorylation.

2. Materials and methods

2.1. Plasmids

The constructs GRE-37TK and 2GRE-37TK which carry one and two copies respectively of the distal GRE located on the promoter of the rat tyrosine aminotransferase gene (TAT) were kindly provided by Dr. W. Schmidt [6]. The GRE in these constructs is placed in front of a minimal thymidine kinase (tk) promoter [18] followed by the coding region of the chloramphenicol acetyl transferase gene (CAT). The construct -37TK carries only the minimal thymidine kinase (tk) promoter followed by the coding region of the CAT gene [6]. The construct MMTV1x carries one copy of the distal GRE located on the mouse mammary tumour virus (MMTV) promoter and was kindly provided by Dr. M. Beato. The construct pRSVGR has the cDNA of rat GR in front of the promoter of Rous sarcoma virus (RSV) [19]. A deletion series of pRShGRa which carries the cDNA of human GR was also used. The deletion mutants are Δ9-385, Δ428-490 and I559. The last construct has a point mutation in the cDNA region that encodes for the C′-terminal part of GR [20]. Finally we used the plasmid pRSVGal with the β-galactosidase gene driven by the RSV promoter.

2.2. Cellular transfection assays

Human hepatoma HepG2 cells were cultured in phenol red-free minimal essential medium (MEM) containing 10% (v/v) fetal calf serum (FCS), 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM glutamine. One day prior to transfection cells were plated in petri

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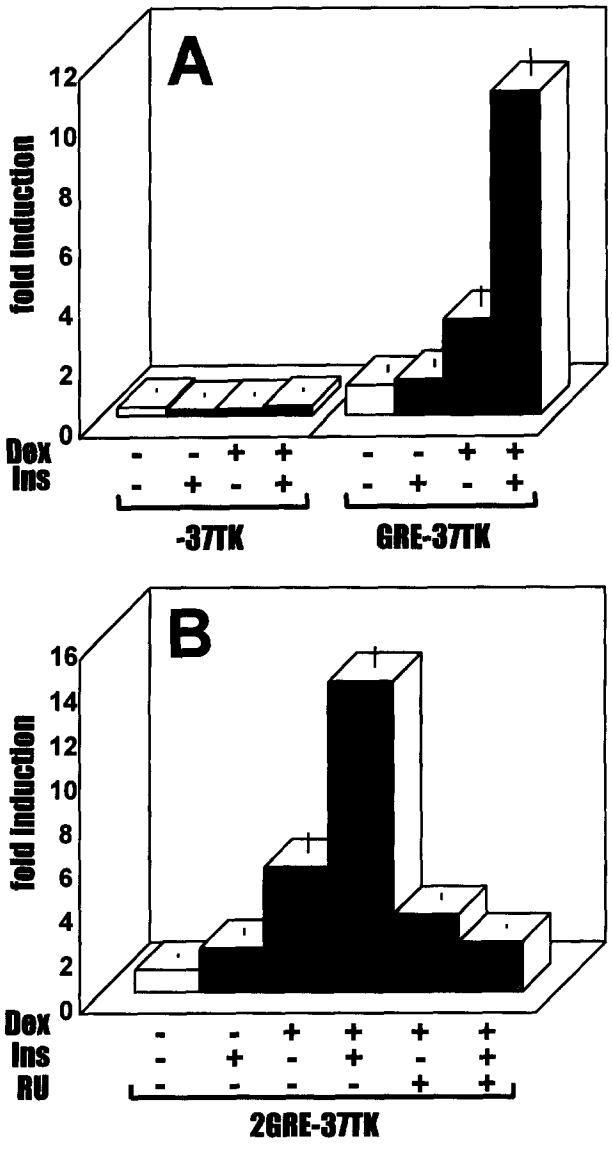

Fig. 1. Effect of insulin and dexamethasone on CAT activity after transfection of HepG2 cells with GRE-37TK and 2GRE-37TK. The cells were transiently co-transfected with 10 μg of the reporter plasmid, 5 μg of pRSVGR and 3 μg of pRSVGal after 24 h incubation in MEM supplemented with 0.5% (v/v) inactivated serum and 1.5% (w/v) BSA. 24 h later the cells were treated with dexamethasone (Dex) insulin (Ins) and the synthetic antiglucocorticoid RU30406 (RU). CAT activity was determined in cell extracts corresponding to 1.5 units of β-galactosidase activity and it was expressed in units. One unit is the CAT activity in the absence of hormones. Values were adjusted to protein concentration measured by Bradford. The reporter plasmids were -37TK and GRE-37TK in (A) and 2GRE-37TK in (B). The reported values are the mean±S.D. of 3 or more individual experiments.

dishes at a density of 1.5×10^6 cells/dish (~50% confluence) in medium with 0.5% (v/v) heat-inactivated and charcoal-stripped FCS supplemented with 1.5% (w/v) BSA. Transfection was carried out by the calcium phosphate co-precipitation technique [21] with minor modifications. 10 μg of reporter plasmid DNA were co-transfected with 5 μg of pRSVGR or deletion mutants per dish. Transfection efficiency was controlled by co-transfection of the internal reference plasmid pRSVGal (3 μg). 4–6 h later the precipitate was removed and the cells were shocked for 30 s with medium containing 15% (v/v) glycerol. The cells were then washed with phosphate-buffered saline and cultured in medium with 0.5% (v/v) inactivated FCS and 1.5% (w/v) BSA. After a 20-h recovery period, hormones were added and allowed to act for another 22 h. Dexamethasone and insulin were added to final concentrations of 1 μM and 7 nM respectively while the synthetic antiglucocorticoid RU30406 was added at a concentration of 10 μM. The cells were harvested by scraping and cellular extracts, prepared by three cycles of freeze-thawing in 0.25 M Tris-HCl (pH 7.8), were heat-treated for 10 min in 60°C in order to inactivate the deacetylase activity [22]. Protein concentration was determined by the method of Bradford and β-galactosidase activity in the extracts, produced by pRSVGal, was used to compensate variations in transfection efficiencies. Mouse fibroblasts (Ltk^-) were transfected as described above in MEM with 10% (v/v) inactivated FCS. The cells were co-transfected with 10 μg of 2GRE-37TK reporter plasmid and 5 μg of pRSVGal as a reference plasmid.

2.3. Electrophoretic mobility shift assay

HepG2 cells were transiently transfected with 5 μg of pRSVGR and 3 μg of pRSVGal and treated with hormones as described above. Cells were washed in phosphate-buffered saline and disrupted in buffer A (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT) by a 5–6 times passage through a 1-ml syringe with a 26-gauge needle. Nuclei were isolated and subsequently resuspended in buffer I (10 mM Tris-HCl pH 8.0, 0.32 M sucrose, 3 mM $CaCl_2$, 2 mM magnesium acetate, 0.1 mM EDTA, 0.1% (v/v) Triton X-100, 1 mM DTT and 2×10^{-4} M PMSF), mixed with two volumes of buffer II (10 mM Tris-HCl pH 8.0, 2 M sucrose, 5 mM magnesium acetate, 0.1 mM EDTA, 1 mM DTT and 2×10^{-4}M PMSF), layered on buffer III (10 mM Tris-HCl pH 8.0, 1.5 M sucrose, 0.1 mM EDTA, 1 mM DTT and 2×10^{-4} M PMSF) and pelleted by centrifugation at $2000\times g$ for 30 min at 4°C. The pellet was washed two times with storage buffer (50 mM Tris-HCl pH 8.0, 25% (v/v) glycerol, 5 mM magnesium acetate, 0.1 mM EDTA, 5 mM DTT and 2×10^{-4} M PMSF) and nuclei were extracted in buffer C (20 mM HEPES pH 7.9, 500 mM KCl, 1.5 mM $MgCl_2$, 25% (v/v) glycerol, 0.2 mM EDTA, 200 μg/ml BSA, 0.5 mM DTT and protease inhibitors: 1 mM leupeptin, 0.2 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml pepstatin and 5 μg/ml antipain) in 4°C for 1 h. The DNA-binding activity of GR in nuclear extracts was analyzed by gel mobility shift assay using as specific DNA a 32-

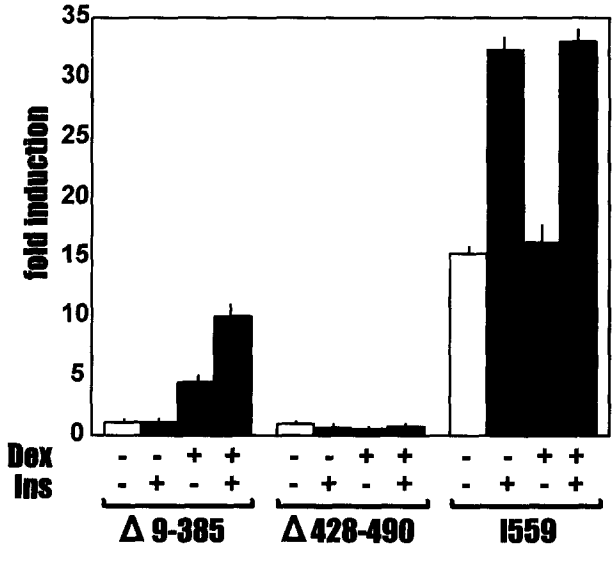

Fig. 2. Effect of GR deletion mutants on the inducibility of dexamethasone and insulin. HepG2 cells were transiently co-transfected with 10 μg of the reporter plasmid 2GRE-37TK, 3 μg of pRSVGal and 5 μg of Δ9-385 or 5 μg of Δ428-490 or 5 μg of I559 after 24 h incubation in MEM supplemented with 0.5% (v/v) inactivated serum and 1.5% (w/v) BSA. 24 h later the cells were treated with dexamethasone (Dex) and insulin (Ins). CAT activity was determined in cell extracts corresponding to 1.5 units of β-galactosidase activity and it was expressed in units. One unit is the CAT activity in the absence of hormones, mediated by the Δ428-490 deletion mutant. Values were adjusted to protein concentration measured by Bradford. The reported values are the mean ± S.D. of 3 or more individual experiments.

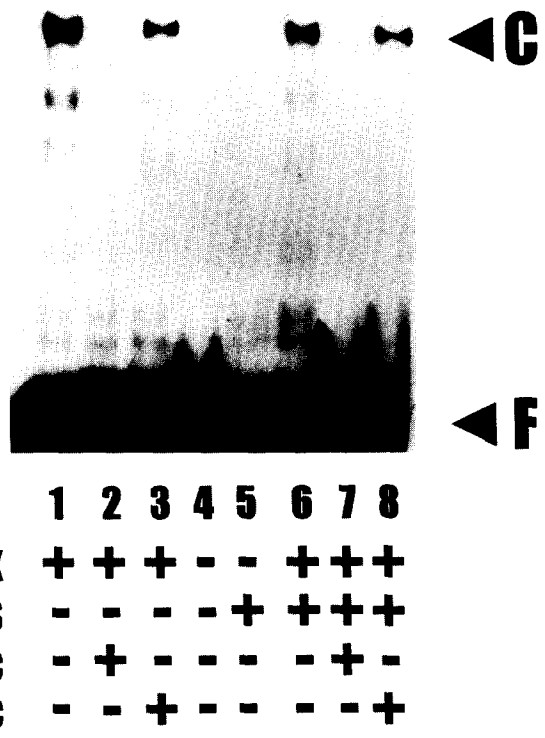

Fig. 3. Effect of insulin on the DNA binding of the GR. HepG2 cells were transiently co-transfected with 10 μg of the reporter plasmid 2GRE-37TK, 5 μg of pRSVGR and 3 μg of pRSVGal after 24 h incubation in MEM supplemented with 0.5% (v/v) inactivated serum and 1.5% (w/v) BSA. 24 h later the cells were treated with 1 μM dexamethasone (lanes 1–3), 7 nM insulin (lane 5) or both (lanes 6–8) and nuclei were isolated and salt extracted. The DNA binding activity of GR in 5 μg of nuclear extract was analyzed by gel mobility shift assay on a 4% non-denaturing polyacrylamide gel using a 3'-end labelled GRE as specific DNA. Competition experiments were carried out with a 500-fold molar excess of unlabelled GRE (sc, specific competitor; lanes 2, 7) or a 500-fold molar excess of an unrelated unlabelled DNA fragment (nsc; non-specific competitor; lanes 3, 8). The gel was dried and autoradiographed.

base pair double-stranded synthetic oligonucleotide corresponding to the most distal (−189 to −162) GRE located on the MMTV promoter (kindly provided by Dr. M. Beato). The GRE was 3'-end labelled with [α-^{32}P]deoxy-CTP by Klenow polymerase. 5 μg of protein from each nuclear extract was incubated in reaction mixture (20 mM Tris pH 8.0, 1.5 mM $MgCl_2$, 0.1 mM, 3 mg/ml BSA, 100 ng/ml poly(dI-dC) and protease inhibitors: 1 mM leupeptin, 0.2 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml pepstatin and 5 μg/ml antipain) for 10 min on ice. After that 20 fmol of labelled GRE were added and the incubation continued for 20 min at 22°C. A 500-fold concentration of unlabelled GRE was used as a specific competitor while a 500-fold concentration of an unrelated DNA fragment was used as a non-specific competitor. Samples were subjected to electrophoresis on a non-denaturing 4% acrylamide gel pre-run for 30 min at 10 mA in Tris-borate buffer (45 mM Tris-borate, 1 mM EDTA pH 8.0). The gel run at 1.5 mA/cm of gel in 4°C and was subsequently dried and autoradiographed. The bands corresponding to GR-GRE complexes were excised and measured for radioactivity in a liquid scintillation counter. Values were adjusted to β-galactosidase activity measured in cell extracts.

2.4. In vivo labelling of GR

HepG2 cells were transiently transfected with 5 μg of pRSVGR as described above. 24 h after transfection the medium was changed to phosphate-free and serum-free MEM (without phenol red) for 1 h. The cells were then metabolically-labelled with 0.2 mCi/ml [^{32}P]orthophosphate (Amersham) for 2 h. The cells were pooled and separated into equal samples. Hormones were added for 10 and 20 min and the incubations were quickly stopped by freezing the cells in liquid nitrogen. The cells were washed with phosphate-free medium supplemented with 10 mM NaF and collected in cold hypotonic buffer A (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 10 mM NaF and protease inhibitors: 1 mM leupeptin, 0.4 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml pepstatin and 10 μg/ml antipain). After 10 min in 4°C the resuspended cells were disrupted and nuclei were isolated and purified as described above. Nuclei were extracted in buffer C (20 mM HEPES pH 7.9, 500 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 10 mM NaF and protease inhibitors: 1 mM leupeptin, 0.4 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml pepstatin and 10 μg/ml antipain) as described above. Insoluble material was pelleted with a centrifugation for 10 min in a microfuge and discarded. After adjusting the KCl molarity to 140 mM, the extracts were precleared by a 1 h incubation with anti-mouse IgG-precoated protein A at 4°C. The protein concentration in the cleared nuclear extracts was determined according to Bradford and aliquots of equal protein amount were incubated overnight with 1 μg of a specific monoclonal anti-GR antibody (MGR3H6) [23] at 4°C. A nuclear extract from cells treated with dexamethasone for 10 min was incubated with antigen-blocked MGR3H6. The immunocomplexes were coupled to protein A-Sepharose which was precoated with anti-mouse IgG, by rotating them for 2 h at 4°C. Immunoprecipitates were washed successively with buffer C (with 140 mM KCl) and eluted in Laemmli sample buffer. After boiling for 5 min, the samples were centrifuged in a microfuge and the supernatants were subjected to electrophoresis on a 8% polyacrylamide gel. ^{14}C-Labelled molecular weight standards were: myosin (M_r 200,000), β-galactosidase (M_r 116,000), phosphorylase *b* (M_r 94,000), bovine serum albumin (BSA) (M_r 67,000), ovalbumin (M_r 43,000).

2.5. Immunoblotting

Electrophoretic gels were transferred to nitrocellulose filters for 4 h at 300 mA. Non-specific antibody binding to the nitrocellulose was prevented by incubating the filter for 1 h at 37°C with block solution (3% (w/v) BSA, 10 mM Tris pH 7.5, 150 mM NaCl, 0.1 mM PMSF). The blot was incubated with the MGR3H6 monoclonal antibody at dilution 1:1000 (v/v) in binding buffer (0.125% (v/v) BSA, 10 mM Tris pH 7.5, 150 mM NaCl, 0.1 mM PMSF, 1% (v/v) Tween 20, 0.04% (v/v) NaN_3) overnight at 4°C. Nitrocellulose was then washed with 10 mM Tris pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween 20 and 0.05% (v/v) NP-40, incubated for 2 h at room temperature with peroxidase-conjugated rabbit anti-mouse IgG at a 1:500 (v/v) dilution and then visualized by incubation with substrate buffer containing 0.18 mg/ml 4-chloro-1-naphthol in 10 mM Tris pH 7.5, 150 mM NaCl, 6% (v/v) methanol and 0.02% (v/v) H_2O_2. The filter was then air-dried and autoradiographed using Fuji X-ray film. Densitometric scanning of the immunoblots was performed with a Molecular Dynamics personal densitometer.

3. Results

3.1. The combinatorial action of glucocorticoid and insulin signalling exerts synergistic effects on the activity of a single GRE

In our previous report we demonstrated that the expression of a glucocorticoid-inducible reporter gene (pMMTV-5A) was potentiated in the presence of both hormones [17]. In this gene the tk promoter was activated by a region of the MMTV-LTR (−362 to −70) which contained four GREs as well as targets for other transcription factors. In this study we are interested in determining the specific target of insulin by which the glucocorticoid induction was enhanced. We started by testing insulin targeting to the transcriptional complex GR-GRE using as reporter genes the GRE-37TK and 2GRE-37TK containing one and two copies respectively of the distal GRE of the rat tyrosine aminotransferase (TAT) promoter in front of a minimal tk promoter [18] and the coding region of the CAT

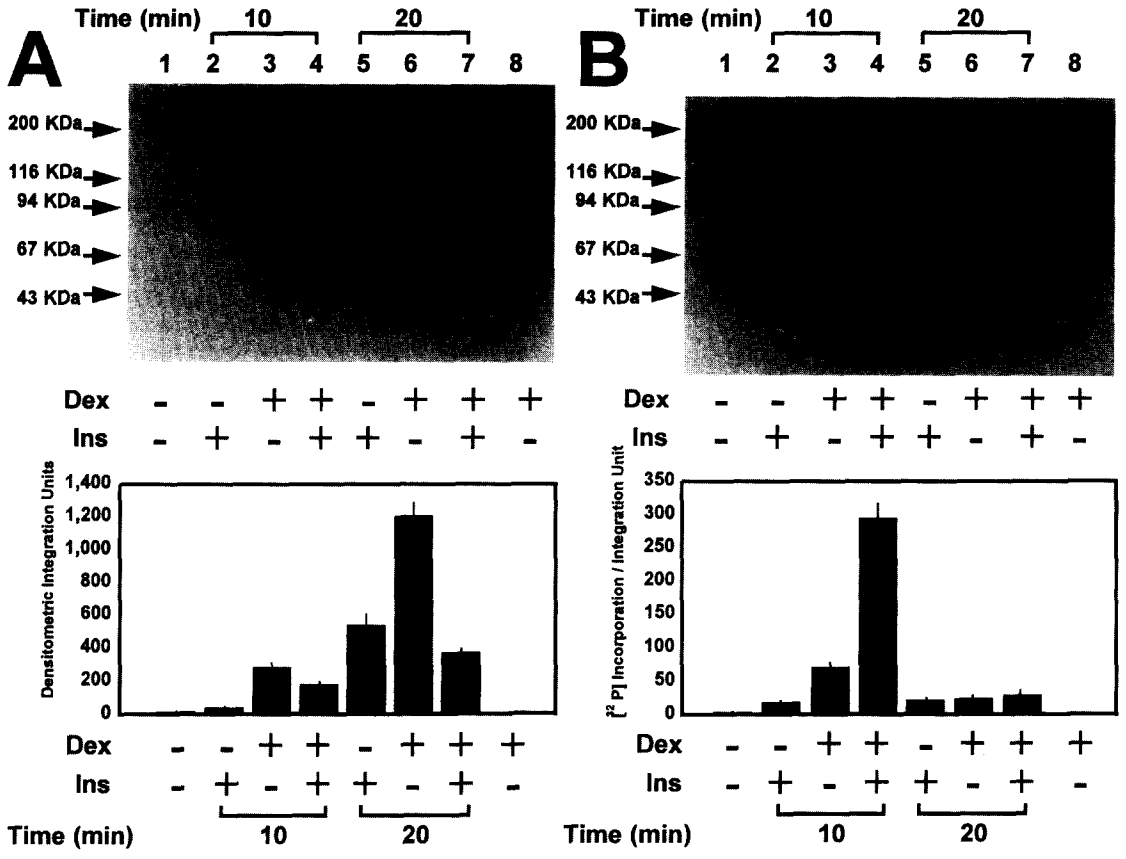

Fig. 4. Hormone-dependent processing and phosphorylation of nuclear-bound GR. HepG2 cells were incubated for 24 h in MEM supplemented with 0.5% (v/v) FCS inactivated and 1.5% (w/v) BSA. The cells were transiently transfected with 5 μg of pRSVGR and 24 h later they were metabolically labelled with 0.2 mCi/ml [^{32}P]Orthophosphate for 4 h in phosphate- and serum-free MEM. After labelling they were treated with 1 μM dexamethasone and 7 nM of insulin for different periods of time. Nuclei were isolated and salt extracted. Receptors from 10 μg of nuclear extract were immunoprecipitated with a specific monoclonal anti-GR antibody (MGR3H6) and subjected to SDS-PAGE. (A — top). The proteins in the gel were transferred to nitrocellulose and the sheet was blotted with MGR3H6. Lane 1 = no hormonal treatment; lanes 2,3,4 = treatment with 7 nM of insulin, 1 μM of dexamethasone and both 7 nM of insulin and 1 μM dexamethasone, respectively for 10 min; lanes 5,6,7 = the same hormonal treatment as in lanes 2,3,4 for 20 min; lane 8 = immunoprecipitation of nuclear receptor with antigen-blocked MGR3H6. (A — bottom) Densitometric scanning was done on immunoblots and protein levels of receptors, at different times of hormonal treatment, were expressed as arbitrary densitometric integration units. The reported values are the mean ± S.D. of 3 individual experiments. (B — top). The nitrocellulose filter was dried and exposed to X-ray film to obtain the autoradiogram. Lane 1 = no hormonal treatment; lanes 2,3,4 = treatment with 7 nM of insulin, 1 μM of dexamethasone and both 7 nM of insulin and 1 μM dexamethasone, respectively for 10 min; lanes 5,6,7 = the same hormonal treatment as in lanes 2,3,4 for 20 min; lane 8 = immunoprecipitation of nuclear receptor with antigen-blocked MGR3H6. (B — bottom) Immunoreactive bands corresponding to GR were excised from the nitrocellulose sheet and counted for radioactivity in a liquid scintillation counter. Values were expressed as ^{32}P-incorporation per arbitrary integration unit. The reported values are the mean ± S.D. of 3 individual experiments.

gene. The reporter genes were cotransfected into HepG2 cells with pRSVGR, a plasmid which carries the cDNA of the rat glucocorticoid receptor, because these cells have extremely low levels of GR ([24] and data not shown). Indeed, the reporter plasmid pMMTV-5A, used for testing the response of HepG2 cells to glucocorticoids, was not activated by dexamethasone (data not shown). As expected, exogenous receptor-mediated activation of GRE-37TK and 2GRE-37TK by hormone was ~3- and ~5-fold, respectively (Fig. 1A and B). In the presence of insulin, induction was enhanced by ~3- and ~2.5-fold, respectively, while insulin alone had a very little effect on the basal CAT activity in either case. Moreover, the synergistic effect of dexamethasone and insulin was significantly inhibited by a 10-fold excess of the anti-glucocorticoid RU30406, demonstrating that this effect requires the presence of active GR (Fig. 1B). The response to insulin observed with transfected GR is identical to the response of endogenous GR, since the synergistic effect was also detected in mouse fibroblasts (Ltk$^-$) or in HTC cells, where the relative abundance of the GR is high (data not shown). As expected, plasmid GRE-37TK produced a weaker induction than the 2GRE-37TK confirming that duplication of the GRE enhances its hormone inducibility [6]. The expression of plasmid -37TK which carries the minimal tk promoter but lacks GREs was not affected by either dexamethasone or insulin alone or by the combination of both hormones (Fig. 1A). These results show that the response of glucocorticoid inducible promoters to both dexamethasone and insulin is mediated by the GRE itself, a fact which requires an active GR.

3.2. *Structural requirements of GR-mediated transcriptional potentiation by insulin*

Since only active GR can mediate the complex effect of both hormones, we used deletion mutants of GR in order to characterize the importance of its functional domains on the phenomenon. We tested the effect of hormones on HepG2

cells transiently cotransfected with reporter plasmid 2GRE-37TK and GR deletion mutants. As Fig. 2 shows, mutant Δ9-385, which lacks the τ1 transactivation domain [25], resulted in a ~2.6-fold enhancement by insulin, of dexamethasone-induced CAT activity, as observed with the wild-type GR (Fig. 1). Mutant Δ428-490, which lacks the DNA-binding domain and glucocorticoid-induced transactivation, was not capable of transactivation in the presence of either dexamethasone alone or both dexamethasone and insulin. Mutant I559, a constitutive transactivator, which lacks part of the steroid binding domain, supported the insulin effect since insulin enhanced constitutive CAT activity by ~2.1-fold (Fig. 2). These results suggest that the N′- and the C′-terminal regions of the GR molecule are not critical for mediating the effect of insulin.

3.3. Insulin does not affect the DNA binding of the GR

In an effort to find if there exists an insulin-dependent, GR-targeted factor which stabilizes GR-GRE interaction, we transiently transfected HepG2 cells with pRSVGR plasmid and treated them with hormones as described in section 2. Nuclei were isolated, salt extracted, extracts were incubated with a synthetic, 3′-end-labelled GRE and DNA–protein complexes were analyzed in non-denaturing polyacrylamide gel (Fig. 3). As we can see in Fig. 3 nuclear GR from dexamethasone-treated cells was capable of binding to its cognate response element (GRE) (lane 1) while no retarded band was observed in the absence of hormones (lane 4). Similarly, no GR-GRE complex was observed with nuclear extracts from insulin-treated cells (lane 5). In the presence of both hormones, neither GR binding to the GRE was enhanced (lane 6) nor any slower migrating bands appeared in the gel. GR binding to labeled GRE was completely abolished in the presence of 500-fold molar excess of specific, unlabelled competitor (GRE) in the reaction mixture (lanes 2 and 7), while a 500-fold molar excess of an unrelated DNA fragment could not prevent formation of the complex (lanes 3 and 8). Thus, insulin does not seem to affect the specific GR-GRE complex that is formed in the presence of dexamethasone. The data exclude the possibility that insulin signalling stimulates a factor to interact directly with the GR or the GR-GRE complex.

3.4. Insulin alters nuclear-bound GR levels and modulates nuclear receptor phosphorylation

That changes in GR functional properties could not account for the increase in GR transactivation by insulin prompted us to search for insulin-induced postranslational modifications of GR. It is well known that GR is a phosphoprotein [26–28] and that insulin induces phosphorylation of various cellular substrates, mediated by its receptor, a protein tyrosine kinase [29]. We decided to examine the effect of insulin in the phosphorylation state of nuclear-bound GR. A detailed time course of changes in receptor phosphorylation and nuclear-receptor levels was conducted following hormone addition. Nuclear-bound receptor levels from HepG2 cells transiently transfected with pRSVGR and metabolically labelled with [^{32}P]orthophosphate were detected by immunoblotting with a highly specific anti-GR monoclonal antibody (MGR3H6) that does not recognize other steroid receptors [23]. Changes in nuclear receptor levels (Fig. 4A) were compared to phosphorylation changes (Fig. 4B) at different times of hormone treatment. In untreated cells, the level of GR phosphorylation was low (Fig. 4B, lane 1) but invariably increased following cell treatment with dexamethasone for 10 min (Fig. 4B, lane 3), while it decreased after 20 min of hormone action (Fig. 4B, lane 6 and bottom). Nuclear-bound GR levels also increased in the presence of dexamethasone to reach a maximum at 20 min (Fig. 4A, lane 6 and bottom). That steroid-induced levels of the nuclear-bound receptor were maximal at 20 min was confirmed by [^{3}H]triamcinolone acetonide (TA) binding experiments (data not shown). Antigen-blocked MGR3H6 could not immunoprecipitate GR (Fig. 4A, lane 8; and Fig. 4B, lane 8). Insulin treatment reproducibly resulted in a 4 ± 0.37-fold (n = 3) increase of the steroid-induced phosphorylation of GR at 10 min (Fig. 4B, lane 4 and bottom) which was not due to elevated nuclear-bound receptor levels. In fact, nuclear-bound GR levels were much lower at 10 min in the presence of both hormones (Fig. 4A, lane 4 and bottom). At 20 min of hormone treatment, ^{32}P-labelling of GR was lower in the presence of both hormones as compared to that in the presence of dexamethasone alone (Fig. 4B, lanes 6 and 7), consistent with the lower levels of nuclear-bound receptor (Fig. 4A, lanes 6, 7 and bottom: and Fig. 4B, bottom). We cannot explain the decrease in nuclear-bound receptor levels when both hormones are present. However, that could be due to rapid GR relocalization process. Thus, the nuclear-bound GR after 10 min of hormone action, is hyperphosphorylated in the presence of insulin. On the contrary, at 20 min of hormone treatment, GR levels are kept low in the presence of both hormones compared to the levels in the presence of dexamethasone alone but the state of GR phosphorylation is not altered.

4. Discussion

In this report we present evidence demonstrating that the functional consequence of the combinatorial action of coupling insulin and glucocorticoid signalling on an single copy of GRE confers synergistic transcriptional transactivation. In agreement with Pan et al. [30], we show here that a single GRE is a target of both hormones, thus excluding the contribution of other DNA binding transcription factors in MMTV-LTR activation by glucocorticoids and insulin [31–33]. Moreover, we show that modulation of glucocorticoid action by insulin requires activated GR since the glucocorticoid antagonist RU40306 inhibited the effect. It is postulated that cellular kinases can modulate the function of the GR by enhancing either its DNA binding ability [34] or its transactivation potential [35–37]. Since insulin activates intracellular signalling which stimulates protein kinases, we examined in more detail its involvement in modulating the GR functional properties. Using cells treated with one or both hormones, we were not able to detect any significant insulin dependent alterations on GR binding to its cognate ligand or to the GRE. Moreover, band shift assays clearly demonstrated that neither the amount nor the mobility of the GR-GRE complex was changed, arguing against the existence of an insulin depended, GR-targeted factor, which stabilizes the GR-GRE interaction. To confirm the above results we studied the effect of GR deletion mutants in response to both hormones.As it is shown (Fig. 2), insulin-mediated increase of transactivation by GR requires neither the C′- nor the N′-terminal portion of the GR protein. It is generally accepted that alterations in nuclear-bound GR levels determine the degree of glucocorticoid-de-

pendent transcription. In this context we examined insulin-mediated changes in glucocorticoid inducibility and nuclear-bound GR levels. Using a monoclonal antibody with high specificity to the rat GR [23], we could detect high levels of nuclear-bound GR after 10 min of steroid treatment, in agreement with previous studies [38]. However, the combination of both hormones produced a dramatic decrease in nuclear-bound GR levels after 10 min of treatment. This apparently contrasts the insulin-induced increase of transactivation by GR. Taking into account that the GR is a phosphoprotein and that, as with several other activators [39], its putative phosphorylation may affect its transcriptional activity, we tested the possibility of insulin induced modifications in the GR phosphorylation state. Indeed, the presence of insulin dramatically increased the dexamethasone-induced phosphorylation of nuclear-bound GR, a fact which was invariably observed after 10 min of hormone treatment. Our results strongly suggest that enhancement of GR transactivation potency by insulin correlates with hyperphosphorylation of nuclear-bound GR. Since the above phenomenon requires the presence of the GR cognate ligand we assume that a sequence of events is taking place. Namely, either insulin phosphorylates GR at critical sites different from those affected by its cognate ligand or a hierarchical phosphorylation occurs in which hyperphosphorylation by insulin is the consequence of the glucocorticoid-induced phosphorylation. Another hypothesis to explain our results is that insulin activates its receptor tyrosine kinase which in turn influences nuclear-targeted signalling responsible for the potentiation of transactivation by GR. Our results represent also a molecular mechanism by which two different signalling pathways can cross-talk in modulating gene expression.

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