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Macromolecular synthesis inhibitors perturb glucocorticoid receptor trafficking

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ABSTRACT

The ability of inhibitors of transcription and translation to prevent glucocorticoid-induced apoptosis has been interpreted to indicate that the cell death machinery requires *de novo* protein synthesis. The transcriptional inhibitors actinomycin D (Act D) and DRB as well as the translational inhibitors CHX and puromycin inhibited early loss of mitochondrial membrane integrity in a dose-dependent manner. This effect was not observed with the transcriptional inhibitor α-amanitin suggesting they may have additional effects. Their role in the glucocorticoid receptor (GR) intracellular trafficking was therefore investigated. Here, we show that Act D and CHX reduced glucocorticoid binding, GR turnover and impaired GR nuclear translocation. We performed the same experiments in different thymocyte subpopulations of Balb/c mice. At the highest dose tested, actinomycin D and cycloheximide abolished glucocorticoid-induced cell death of CD4+CD8+ and CD4+CD8−. In all subsets, Act D, DRB, as well as CHX and puromycin prevented receptor nuclear translocation, indicating a general alteration of GR trafficking. Overall, our data support a direct effect of macromolecular inhibitors on GR activation and trafficking. Finally, direct alterations of the functional properties of the glucocorticoid receptor might be responsible for cell death prevention by actinomycin D, DRB, cycloheximide and puromycin.

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1. Introduction

Glucocorticoid-induced apoptosis of thymocytes was one of the first described programmed cell death [1] but mechanisms by which glucocorticoids (GC) elicit cell death are still unresolved [2]. At the molecular level, GC binds to the glucocorticoid receptor (GR), a member of the nuclear receptor family. GR is primarily located in the cytoplasm as large complexes with heat shock proteins and chaperones which are required for proper steroid binding [3]. Hormone binding triggers GR translocation to the nucleus where the GC/GR complex transactivates or represses gene expression through glucocorticoid response elements (GRE) [4]. Transrepression results from direct or indirect interference with several transcription factors such as NF-κB and AP-1 at both the cytoplasmic and nuclear levels [5]. GC also have rapid non-genomic effects, observed within seconds or minutes [6]. These effects are too fast to be regulated by transcriptional and translational steps. They involve generation of second messengers (Ca++, IP3, DAG or AMPc) and GR-interacting protein kinases and phosphatases [6–8].

GC-induced cell death involves cascades of events, including mitochondrial permeability transition, cytochrome c release, caspase activation and DNA fragmentation [2]. However, initiating events are still a matter of controversy. In several studies, *de novo* RNA and protein synthesis have been found to be required since actinomycin D (Act D) and cycloheximide (CHX), respective inhibitors of transcription and translation, prevent dexamethasone-induced apoptosis [9]. This scheme seems further validated by recombinant mice expressing a mutated GR devoid of dimerization property (GR^{dim}) that retains transrepressive but no transactivating and apoptotic activities [10]. This work validates the model by which GC induce the expression of "death genes" although such genes have not been found at the moment [2,9]. On the other hand, results obtained with GR mutants displaying reduced transactivation activity support the idea that GC-induced apoptosis *in vitro* is restricted to the transrepressive activity of the glucocorticoid receptor on "survival genes" [11,12].

This scheme is rendered even more complex by the use of mouse thymocytes, a widely used physiological model of glucocorticoid-induced cell death due to its extreme sensibility to corticoids. The thymus provides an inductive environment for development of T-lymphocytes from hematopoietic progenitor cells, which are

Abbreviations: Act D, actinomycin D; CHX, cycloheximide; Dex, dexamethasone; DN, double negative cells; DP, double positive cells; DRB, 5,6-dichlorobenzimidazole 1-β-D-ribofuranoside; GC, glucocorticoid; GR, glucocorticoid receptor; GRE, glucocorticoid response element; SP, single positive cells; $\boldsymbol{\Delta\Psi_m}$, mitochondrial membrane potential.

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critical cells of the adaptive immune system. Thymocytes are divided in four subsets, the single positive (SP) CD4+ or CD8+ cells, the double negative (DN) CD4−CD8− and double positive (DP) CD4+CD8+ cells. Significant differences of GR expression were observed in these subsets [13]. The highest GR expression was found in DN thymocytes, and decreased during development via the CD8+ subpopulation into the DP subset [13,14]. Interestingly, the latter population, although expressing the lowest GR level was the most sensitive to GC-induced apoptosis [13], raising the possibility that apoptosis may not be induced by transcription regulation but by non-genomic pathways [15]. Additionally, mitochondrial GR translocation in this DP population was found to correlate with their sensitivity to glucocorticoid-induced apoptosis [16]. Taken together, these data indicate that the exact role of the glucocorticoid receptor and the subsequent activation of the apoptotic pathway are far from being fully elucidated and may also depend on the cellular context.

The assumption that glucocorticoid-induced apoptosis requires *de novo* protein synthesis has been previously re-evaluated in thymocytes, the most physiological model [17]. Studies with rat thymocytes indicated that DNA cleavage induced by thapsigargin, methylprednisolone, a synthetic glucocorticoid, and ionomycin were inhibited by cycloheximide and emetine but not by puromycin at concentrations that reduced protein synthesis by more than 80% [17], demonstrating that the degree of translational blockade induced by these inhibitors did not correlate with their protective effect and suggesting they could delay the onset of apoptosis rather than prevent it. Interestingly, drugs known to interfere with GR macromolecular complexes stability, such as geldanamycin, alter GR nuclear translocation, transactivation and transrepression activities and also inhibit glucocorticoid-induced apoptosis [18–21]. Taken together, these data suggested cycloheximide and actinomycin D might affect glucocorticoid receptor trafficking, thus preventing cell death. Therefore, cellular localization of glucocorticoid receptor in all thymocyte subsets, free or steroid-bound was carried out in the presence or the absence of macromolecular inhibitors: cycloheximide, DRB, actinomycin D or puromycin. Our data suggest that alteration of the functional properties of the glucocorticoid receptor, rather than inhibition of GR-induced death genes expression, might be responsible for cell death prevention by macromolecular inhibitors and indicate that the exact role of these inhibitory drugs is far more complex than initially conceived and depends on the cellular context.

2. Materials and methods

2.1. Reagents

Non-radioactive dexamethasone, cycloheximide, alpha-amanitin, 5,6-dichlorobenzimidazole 1-β-D-ribofuranoside (DRB), puromycin dihydrochloride were purchased from Sigma. Actinomycin D was purchased from Calbiochem. [6,7-3H]-Dexamethasone ([^{3}H]-Dex; 35–50 Ci/mmol) was purchased from Perkin Elmer (Zaventem, Belgium). Stock solutions of alpha-amanitin and puromycin were made in H_2O while dexamethasone, DRB, Act D and CHX were prepared in ethanol.

2.2. Thymocytes and splenocytes preparation and culture

Thymocytes were prepared from 5 to 8 weeks old Balb/c mice as described [22]. Cells were cultured at 10×10^6 cells/ml in RPMI medium without phenol red (Gibco BRL), supplemented with 10% heat-inactivated and charcoal-stripped foetal calf serum, 1000 UI/ml penicillin, 1000 μg/ml streptomycin and incubated at 37 °C in 5% CO_2. Dexamethasone was used at 10^{-7} M for 1 or 4 h (see details in the legends).

2.3. Flow cytometry analysis

Mitochondrial membrane potential and plasma membrane permeabilisation were assessed by flow cytometry analysis using CMXRos fluorescent dye and the impermeant DNA intercalatant YOPRO-1, respectively (Molecular Probes) [23,24]. Briefly, cells (5×10^5) were incubated with CMXRos (50 nM) and YOPRO-1 (200 nM) for 30 min in culture medium at room temperature in the dark and analyzed by flow cytometry (EPICS XL or CYAN, Beckman Coulter) using Expo 2 or Summit analysis softwares. High CMXRos and low YOPRO-1 cells were considered as living cells whereas low CMXRos and high YOPRO-1 cells were considered as apoptotic cells.

Immunophenotyping was carried out using the CYAN apparatus (Beckman Coulter), using PE rat anti mouse CD4 (IgG2b, κ) and APC rat anti mouse CD8 (IgG2a, κ) monoclonal antibodies and their matching isotypes (Becton Dickinson) to evaluate non specific binding. Briefly, cells (5×10^5) were incubated with CMXRos (50 nM) for the last 30 min in culture medium and then stained with isotypes or specific antibodies (500 ng/ml) in the presence of excess FcR block (Miltenyi) for 15 min before analysis.

2.4. Protein extraction

Cells (30×10^6) were washed twice with cold PBS by centrifugation at $750 \times g$ for 10 min at 4 °C and lysed in 150 μl of RIPA buffer (10 mM Tris–HCl, 140 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 2 mM PMSF, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 10 μg/ml leupeptin, pH 7.5). After 1 h on ice, the lysate was centrifuged at $15{,}000 \times g$ for 15 min at 4 °C and the supernatant was collected. Protein concentration was determined by Bradford assay.

2.5. Isolation of cytosol and nuclear fractions

Thymocytes were washed with PBS and resuspended in 500 μl ice-cold buffer A (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 2 mM DTT, 0.5 mM PMSF, 5 μg/ml pepstatin, 5 μg/ml aprotinin, 5 μg/ml leupeptin, pH 7.8) by gentle pipetting. The cells were allowed to swell on ice for 15 min. 25 μl of 10% NP-40 in buffer A was added and the tubes were vortexed for 20 s. The homogenate was centrifuged for 2 min at $1400 \times g$ at 4 °C. The resulting cytosolic supernatant was removed and the nuclear pellet was resuspended in 50 μl ice-cold buffer C (20 mM HEPES, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 25% glycerol, 2 mM DTT, 0.5 mM PMSF, 5 μg/ml leupeptin, pH 7.9) for 30 min on ice. The nuclear extract was centrifuged for 5 min at $14{,}000 \times g$ at 4 °C and protein content of cytosol and nuclear fractions was determined by Bradford assay.

2.6. Western blotting

Equivalent amounts of protein were electrophoresed on SDS-polyacrylamide gels and electroblotted onto nitrocellulose membrane (Amersham Biosciences). Membranes were blocked and probed with appropriate primary antibodies: GR (M-20, sc-1004), lamin B (sc-6217) (Santa Cruz Biotechnology) and β-actin (A-5441, Sigma). Primary antibodies were detected by appropriate horseradish peroxidase-conjugated secondary antibody and visualized using the enhanced chemiluminescence detection system (Amersham Biosciences).

2.7. Immunofluorescence experiments

Cells (5×10^5) were centrifuged ($200 \times g$ for 10 min) on cytospin slides, fixed with methanol for 20 min at −20 °C and permeabilized with 0.1% Triton X-100 for 10 min and washed again with

PBS. The slides were then blocked for 30 min with 3% BSA and incubated overnight at 4 °C with the anti-GR antibody (M-20, sc-1004, Santa Cruz Biotechnology) in 3% BSA. After washing, cells were stained with a FITC-conjugated anti-rabbit IgG antibody (Jackson Immunoresearch) in 3% BSA for 1 h at 37 °C, washed with PBS and nuclei were stained by propidium iodide or Hoechst 33258. Cells were examined by confocal microscopy (Leica or Zeiss 710).

For cell surface staining, the protocol of Wiegers et al. [13] was used with slight modifications. Cells were stained with CD4-PE and CD8-APC (or matching isotypes) as for flow cytometry in the presence of FcR block for 15 min at room temperature. Cells were washed twice in PBS/3% BSA. Subsequent intracellular staining of GR was carried out after cell fixation with 2% PFA for 30 min at room temperature, washed and permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature. GR staining was performed as described above.

2.8. Binding assays

Whole cell binding assays were performed in duplicate as previously described with minor modifications [25]. Cells (10^7 thymocytes/ml) that had been pretreated for 30 min with solvent, CHX (5 μM) or Act D (1 μg/ml) were resuspended in RPMI buffer containing various concentrations of [^{3}H]-dexamethasone in the absence (uncompeted) or presence (competed) of 500-fold molar excess of unlabeled Dex. Cells were incubated for 1 h at 37 °C in a humidified CO_2 incubator and then washed three times with ice-cold phosphate buffered saline (PBS) and subsequently resuspended in 100 μl PBS. The bound radioactivity in 50 μl aliquots was then quantified by liquid scintillation spectroscopy. The amount of specific steroid cell binding was calculated by subtracting nonspecific (competed) from total (uncompeted) binding.

2.9. Statistical analysis

Experimental results are expressed as means ± S.E. of four separate replicate experiments. Levels of significance were evaluated by Student's *t*-test, and $p < 0.05$ was considered significant using Graphpad Prism (San Diego, CA).

3. Results

3.1. Macromolecular synthesis inhibitors inhibit glucocorticoid-induced apoptosis

Glucocorticoid-induced cell death is described to depend on *de novo* transcription and translation since specific inhibitors such as actinomycin D (Act D) or cycloheximide (CHX) inhibited dexamethasone (Dex)-induced apoptosis [2,9]. We therefore used three others molecules, DRB and alpha-amanitin as inhibitors of transcription and puromycin as inhibitor of translation to assess their efficacy in this model. Since mitochondria have been extensively studied in this model and found to be early involved [26], the ability of these compounds to inhibit apoptosis was first assessed studying early loss of mitochondrial potential. Treatment with dexamethasone for 4 h induced a potent loss of mitochondrial membrane potential ($\Delta\Psi_m$), reflecting the early mitochondrial membrane deregulation (Fig. 1A) as previously reported [26,27]. The results obtained after cytometric analysis at the maximal dose tested for Act D (1 μg/ml) and CHX (5 μM), doses at which we observed a complete inhibition of GR-induced transcription and translation (data not shown), indicate that these two compounds are not toxic (Fig. 1A left panels) and completely inhibit Dex-induced apoptosis (Fig. 1A right panels). Fig. 1B summarizes the data obtained using the five drugs tested in a dose-dependent manner. Inhibitors of transcription such as Act D and DRB inhibited cell death in a dose

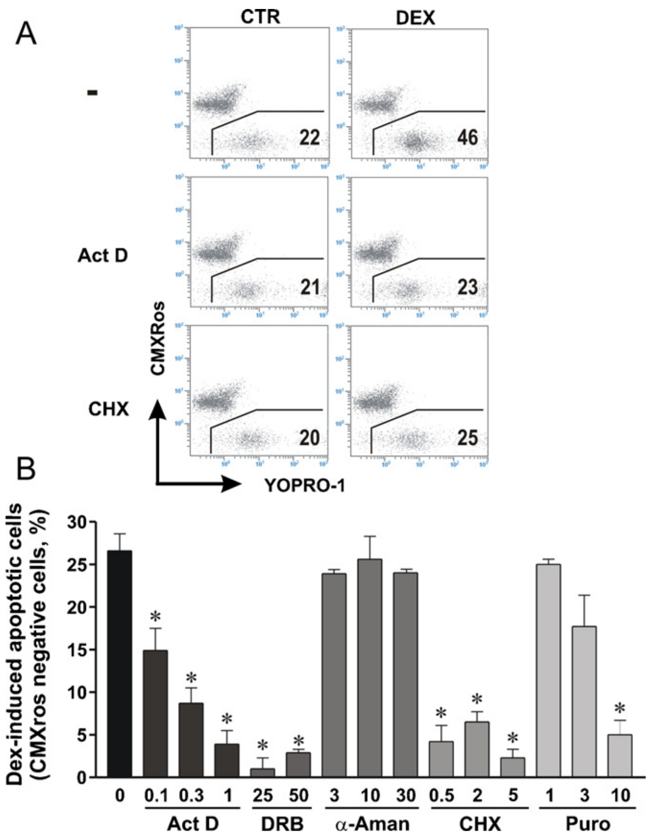

Fig. 1. Effect of macromolecular synthesis inhibitors on glucocorticoid-induced apoptosis. Thymocytes were pretreated for 30 min with actinomycin D (Act D, 1 μg/ml) or cycloheximide (CHX, 5 μM) and then were incubated in the absence (CTR) or presence of dexamethasone at 10^{-7} for 4 h. (A) Early apoptosis was evaluated by biparametric analysis after CMXRos and YOPRO-1 staining. (B) Thymocytes were treated as above with different doses of Act D (μg/ml), DRB (μM), α-amanitin (μg/ml), CHX (μM) and puromycin (μg/ml) and then in the absence or presence of dexamethasone at 10^{-7} for 4 h. Loss of mitochondrial potential ($\Delta\Psi_m$) was assessed by CMXRos staining. The results are the mean from three independent experiments. (*$p < 0.05$, Student's *t*-test).

dependent manner. However, alpha-amanitin, while not toxic per se, was completely ineffective to block Dex-induced cell death in the range 3–30 μg/ml, suggesting transcription inhibitors Act D and DRB may have additional effects. Translation inhibitors CHX and puromycin also displayed a dose-dependent ability to inhibit loss of $\Delta\Psi_m$.

Our data confirmed that macromolecular inhibitors, at doses routinely used to assess the role of transcription and translation, are potent inhibitors of Dex-induced cell death. However, results obtained with α-amanitin suggest they may have uncharacterized additional effects.

3.2. Macromolecular synthesis inhibitors perturb GR trafficking

Alteration of intracellular trafficking has already been reported for estrogen and progesterone receptors, two members of the nuclear receptor family, upon macromolecular synthesis inhibitors [28–32]. We therefore checked for glucocorticoid receptor subcellular localization upon Dex treatment in the presence or absence of both transcription and translation inhibitors. Western blotting of actin and lamin B, proteins restricted in the cytoplasm and nucleus, respectively, did not demonstrate any cross-contamination and validated our protocol (Fig. 2A). As expected, in the absence of steroids, the glucocorticoid receptor was mainly located in the cytoplasm. Thymocytes treated with Dex for 1 h showed little GR in the cytoplasmic fraction and high levels of protein in the nuclear

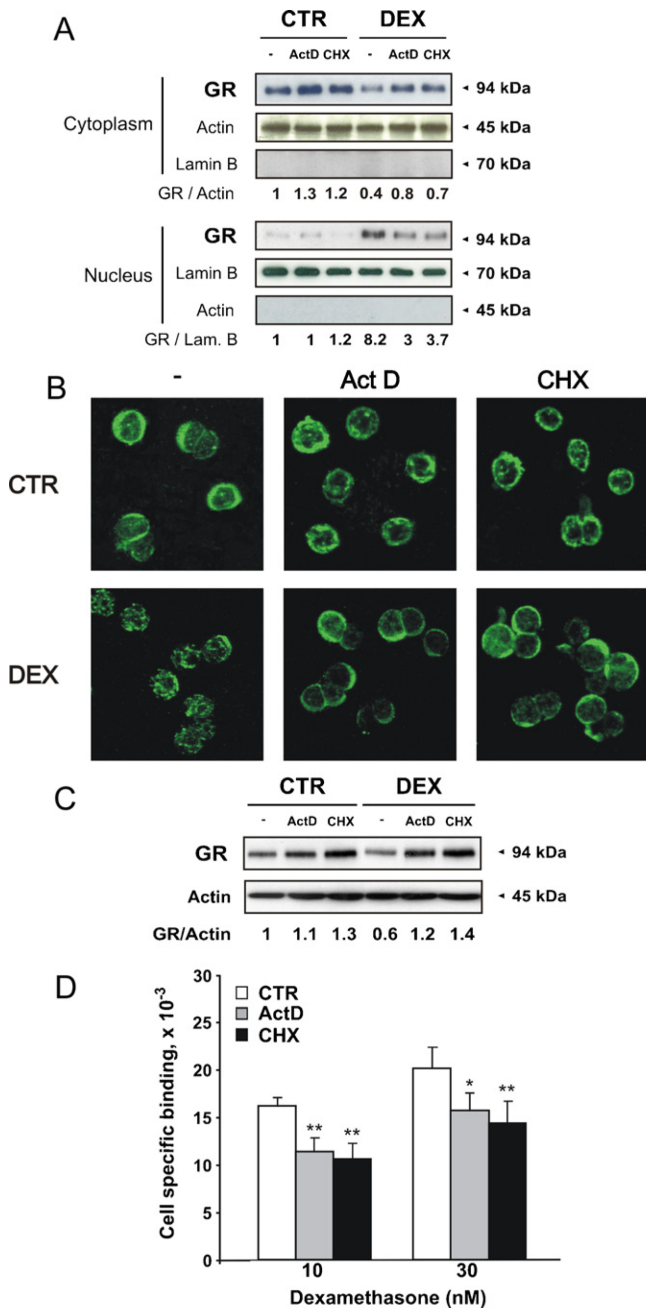

Fig. 2. Act D and CHX disturb GR intracellular trafficking and affect GR whole cell binding. Thymocytes were pretreated for 30 min with actinomycin D (Act D, 1 μg/ml) or cycloheximide (CHX, 5 μM) and then were incubated in the absence (CTR) or presence of dexamethasone at 10^{-7} M for 1 h (A, B and D) or 4 h (C). (A) Glucocorticoid receptor localization was analyzed by Western blotting on cytoplasmic and nuclear extracts. Actin and lamin B were used as loading control for cytoplasmic and nuclear extracts, respectively. Blots for lamin B and actin in cytoplasmic and nuclear extracts, respectively, were overexposed and were used as control for cellular fractionation quality. GR/actin and GR/lamin B ratios were calculated to quantify changes in GR localization. (B) Glucocorticoid receptor localization was analyzed by confocal microscopy. (C) Dex-induced receptor degradation was analyzed by Western blotting on total protein extracts. Actin was used as loading control and GR/actin ratio was calculated to quantify GR expression. (D) Thymocytes (10^7/ml) were pretreated for 30 min with actinomycin D (Act D, 1 μg/ml) or cycloheximide (CHX, 5 μM) and then were incubated in the absence (CTR) or presence of 10 or 30 nM [^{3}H]-Dex in the presence or absence of 500-fold excess unlabeled Dex for 1 h. The subsequent whole-cell specific binding of [^{3}H]-Dex was quantified as described in Section 2. Each bar represents the mean of quadruplicate binding assays performed four times (*$p < 0.05$ and **$p < 0.01$, Student's *t*-test).

fraction (Fig. 2A). Surprisingly, thymocytes pretreatment with Act D or CHX limited nuclear entry of the receptor which remained preferentially in the cytoplasm (Fig. 2A). Confocal microscopy was therefore carried out to clarify the effects of Act D or CHX on GR localization (Fig. 2B). Parallel to Western blot data (Fig. 2A), GR was mainly located in the cytoplasm in control cells and became nuclear upon Dex treatment. When Dex was added to thymocytes pretreated with Act D or CHX, the receptor mainly remained in the cytoplasm, suggesting its functions were altered. These data strongly suggest that a possible explanation for Act D and CHX inhibition of corticoid-induced apoptosis in thymocytes could be a loss of function of GR within the cytoplasm due to an altered cytoplasmic receptor activity. In consequence, we examined the possible effect of these drugs on GR activation and binding capacities. A well described consequence of GR activation by ligand is the subsequent degradation by proteasome [33]. As seen in Fig. 2C, Dex treatment induced GR degradation, measured as 40% of total GR. Act D and CHX alone increased the total amount of glucocorticoid receptor as compared with control cells (Fig. 2C). Steroid addition did not modify the phenomenon, suggesting Act D and CHX may interfere with GR activation and proteasomal turnover (Fig. 2C). At this stage, Dex-induced nuclear entry of the glucocorticoid receptor was impaired (Fig. 2A and B), suggesting it was in a non-responsive state. Therefore, we examined the possible effect of these drugs on GR binding capacities in whole cells using [^{3}H]-dexamethasone at two different concentrations (10 and 30 nM). Pretreatment for 30 min with Act D or CHX induced a modest but statistically significant decrease (<30%) of Dex specific binding as compared to control cells binding capacity (Fig. 2D). These data suggest that the reduced binding properties of GR treated with CHX or Act D do not correlate with the dramatic effect of these drugs on the inhibition of Dex-induced apoptosis, indicating other mechanisms may be responsible for the effect.

Taken together, these data strongly suggest that a possible explanation for Act D and CHX inhibition of corticoid-induced apoptosis in thymocytes could be a loss of function of GR within the cytoplasm due to an altered cytoplasmic receptor activity rather than a consequence of direct inhibition of GR-induced transcription or translation.

3.3. Variability of macromolecular synthesis inhibitors to perturb GR nuclear entry

As shown in Fig. 2B, Act D and CHX prevented liganded GR to enter the nucleus. Profiling of GR-associated fluorescence was plotted and used to examine the effect of various inhibitors on GR shuttling. A typical example is provided with Act D (1 μg/ml) (Fig. 3A). In control cells, the drug affected GR mobility in the cytoplasm, with redistribution to the periphery of the nucleus for 30% of observed cells, indicating that the drug itself was able to destabilize the GR microenvironment. Pre-treatment with Act D prevented GR nuclear translocation after Dex addition. Fig. 3B and C summarizes the data obtained for thymocytes and splenocytes, respectively. Act D and DRB transcription inhibitors were used at the doses used to check for their ability to inhibit Dex-induced thymocyte apoptosis. Act D retained GR in the cytoplasm in a dose-dependent manner that exactly reflected its capacity to inhibit apoptosis (Fig. 1B). DRB used at the highest dose (50 μM) only partially (50%) inhibited GR-Dex complex entry in the nucleus. Similar results were obtained using the translation inhibitors CHX and puromycin, respectively (Fig. 3B). CHX inhibited GR nuclear entry following Dex-treatment in a dose-dependent manner that did not correlate with its ability to inhibit apoptosis at 2 μM (Fig. 1B). We then used more differentiated primary cells such as splenocytes. As shown in Fig. 3C, GR was more restricted in the cytoplasm in basal conditions as compared to thymocytes (72% vs 60.2%, respectively) while Dex induced a

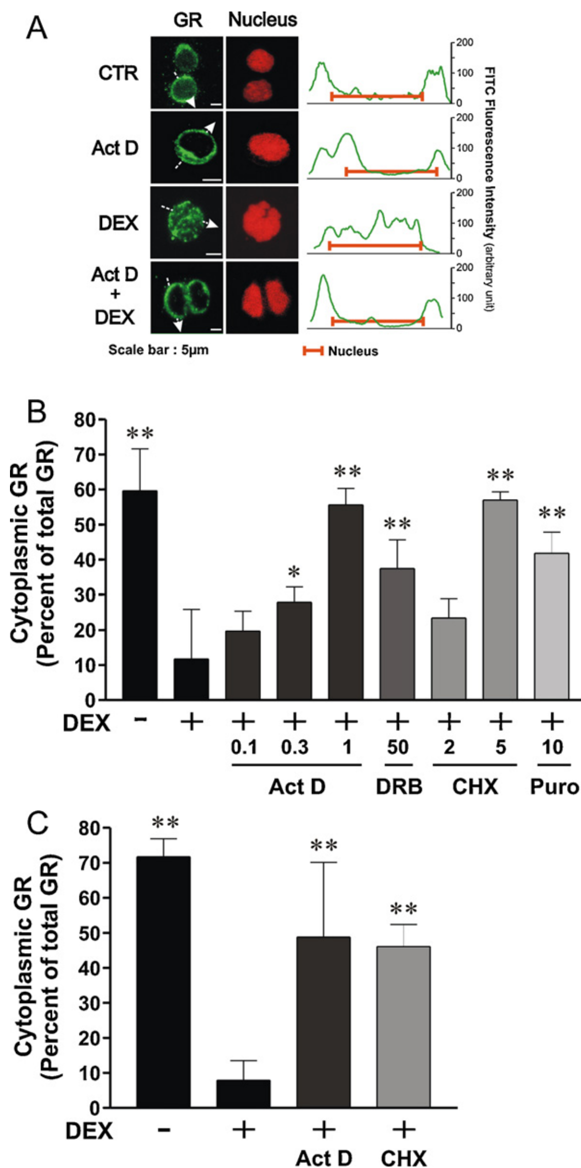

Fig. 3. Macromolecular inhibitors disturb GR nuclear translocation. Thymocytes were pretreated for 30 min with different concentrations of Act D (μg/ml), DRB (μM), α-amanitin (μg/ml), CHX (μM) and puromycin (μg/ml) and then in the absence or presence of Dex at 10^{-7} M for 1 h. (A) Panels represent GR fluorescence intensity profiles of corresponding cell cross-sections symbolized by the dotted arrows. Nuclei were counterstained by propidium iodide. Red horizontal bars on the right panel represent nucleus position. (B) From fluorescence intensity values as shown in (A right panel), cytoplasmic GR was calculated as a percent of total GR (cytoplasmic plus nuclear GR fluorescence intensities). Bars represent the mean ± SEM measured in thymocytes of three independent experiments. (C) Same as in (B) for splenocytes. All values were compared to cytoplasmic GR in Dex-treated cells for statistical analysis (* $p < 0.05$ and ** $p < 0.01$, Student's *t*-test). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

more important nuclear GR entry (8.9% vs 12%, respectively), suggesting GR shuttling may differ in splenocytes and in thymocytes. Here again Act D and CHX at the higher dose inhibited GR nuclear entry in splenocytes (Fig. 3C), but to a lesser extent than they did in thymocytes (Fig. 3B).

We also used a human epithelial carcinoma cell line (HeLa cells) and found that neither Act D nor CHX inhibited GR nuclear entry in response to Dex (data not shown). Taken together, these results suggest that prevention of GR nuclear translocation by macromolecular inhibitors is not a general mechanism but seems restricted to thymocytes and splenocytes.

3.4. Effect of macromolecular synthesis inhibitors on GC-induced apoptosis in thymocyte subsets

It has been previously established that all thymocyte subsets express GR with a degree that does not correlate with the ability of the complexed GR to induce apoptosis [13], the double positive CD4+CD8+ subset (DP), which has been shown to be most sensitive to GC-induced apoptosis, expressing the lowest GR amount [13,14]. We then checked which thymocyte subsets were targeted by macromolecular inhibitors. We first determined sensitivity to GC of the various subpopulations by incubating thymocytes with Dex for 4 h followed by staining with anti-CD4, anti-CD8a and CMXRos. As shown in Fig. 4A, CMXRos staining was first plotted and thymocyte subsets were analyzed on apoptotic and live subpopulations. In our mouse model, basal apoptosis of double negative (DN) and CD8 subsets was high while apoptosis of DP and CD4 subsets was low. Our results confirmed previously published data and showed that DP cells were the most sensitive to GC-induced apoptosis, whereas the DN and the CD8+ subsets appeared to be resistant (Fig. 4A and B).

Having established GC-induced apoptosis in various thymocyte subsets, we wondered how Act D and CHX affected thymocyte subsets following Dex treatment. Fig. 4A exemplifies the results obtained with the two drugs in the presence or absence of corticoids, whereas Fig. 4B recapitulates the percentages of apoptosis in each subset. Act D was not toxic at the highest dose tested and completely inhibited cell death of the more represented DP and CD4+ subsets. As for Act D, CHX completely abrogated loss of $\Delta\Psi_m$ in the DP and CD4+ subsets (Fig. 4B).

3.5. Confocal laser microscopy of GR in thymocyte subpopulations

Finally, to characterize the effect of macromolecular inhibitors on GR localization in thymocyte subsets, aliquots of thymocytes were stained for CD4, CD8, GR and DNA and analyzed by confocal microscopy [13]. Fig. 4C shows examples of anti-GR stained cells expressing CD4 and/or CD8 or DN cells. Our results show that DP cells express the lowest GR amount, which was consistent with previous reports [13,14]. Dex induces a nuclear GR distribution in all thymocyte subsets as a consequence of GC–GR binding. Fig. 4C depicts the nuclear GR distribution in DP and DN cells. Similar localization was found for mature CD4+ and CD8+ subsets (not shown). In presence of Act D (1 μg/ml) and DRB (50 μM) macromolecular inhibitors, GR was refractory to Dex treatment and remained in the cytoplasm of all thymocyte subsets. Similar results were obtained with CHX (5 μM) and puromycin (10 μg/ml) (not shown), suggesting these macromolecular inhibitors acted through a common general mechanism in the thymocyte population.

4. Discussion

The molecular events leading to glucocorticoid-induced cell apoptosis are still unclear. Investigation of the precise role of the glucocorticoid receptor did not solve the problem. Studies using a mutated receptor devoid of dimerization properties, a hallmark of receptor transcriptional capacity, described a lack of apoptotic activity after glucocorticoid treatment, suggesting transcription was necessary in the cell death process [10]. These results have been counterbalanced by several studies using mutated receptors harbouring a point mutation or lacking most of the transcriptional activation signal [12,34,35]. These data suggested that glucocorticoid-induced cell death may not necessarily require gene transcription and subsequent translation of the gene products. Additionally, increasing evidences are in favour of non-genomic GR signalling [15].

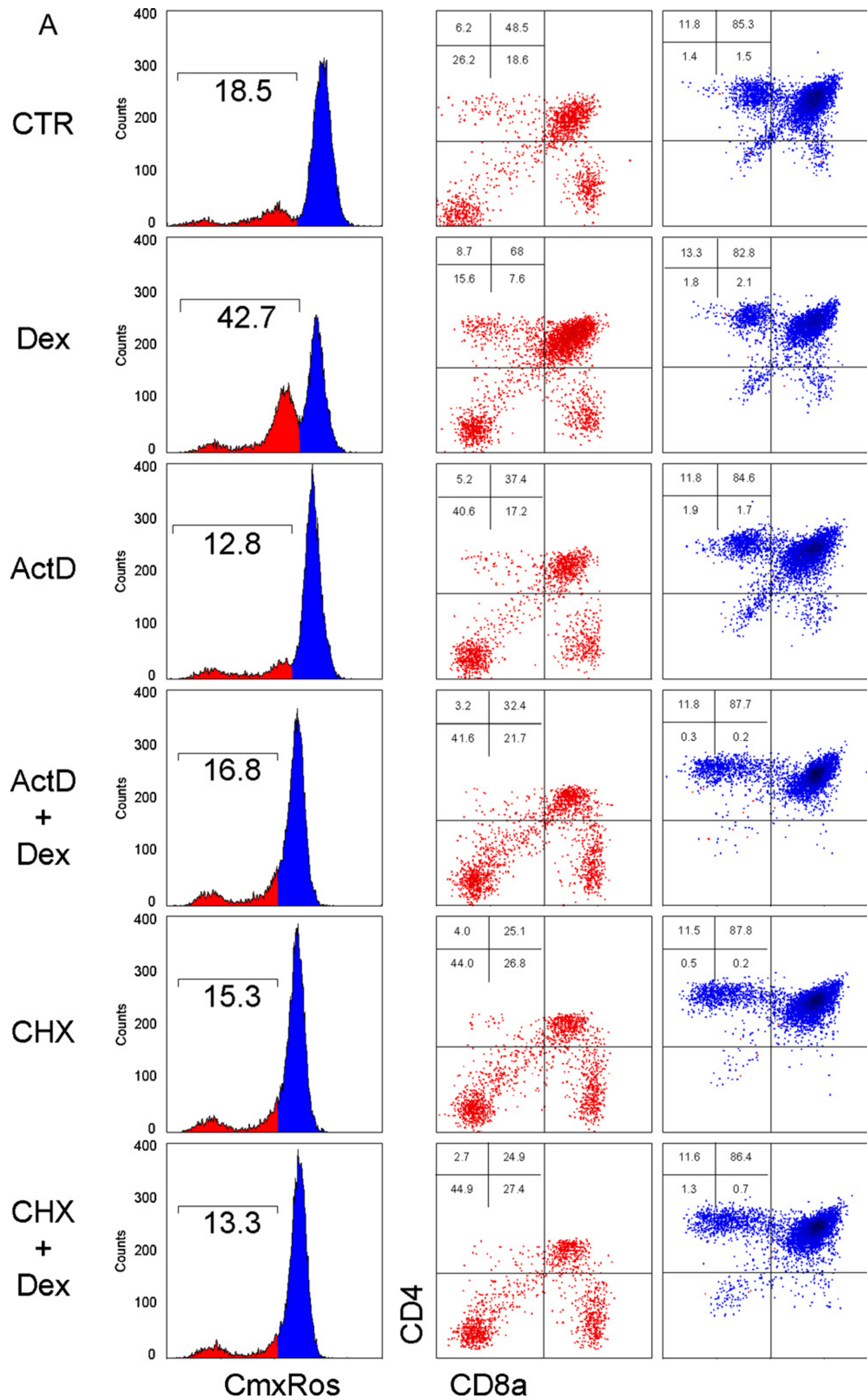

Fig. 4. Effect of macromolecular inhibitors on Dex-induced apoptosis and GR localization in thymocyte subsets. Thymocytes were pretreated for 30 min with Act D (1 μg/ml) or CHX (5 μM) and then were incubated in the absence (CTR) or presence of dexamethasone at 10^{-7} for 4 h (A and B) or 1 h (C). Cells were then stained with anti-CD4-PE and anti-CD8-APC (or matching isotypes) and CMXRos to assess the mitochondrial potential, as described in Section 2. (A) Plots in the left column show the apoptotic (low $\Delta\Psi_m$, red) and live (high $\Delta\Psi_m$, blue) thymocytes for each condition. Percentage of total apoptotic cells is indicated in each plot. Dot plots in the two right columns show the changes on the thymocytes subsets with CD4/CD8 staining in apoptotic (red) and live (blue) cells. Numbers in quadrants correspond to percentages of thymocyte subsets for each condition in apoptotic and live cells. (B) Percentages of apoptotic cells for each thymocyte subset was deduced from Fig. 4A and plotted. Values are representative of three independent experiments. (C) Confocal laser microscopy analysis of GR in thymocyte subsets. Thymocytes were treated as in Fig. 4A except for Dex incubation that lasted only 1 h. Thymocytes were then stained with CD4, CD8, GR and DNA as described in Section 2. *, CD4+CD8+ double positive cell and **, CD4−CD8− double negative cell. Results shown are representative of three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

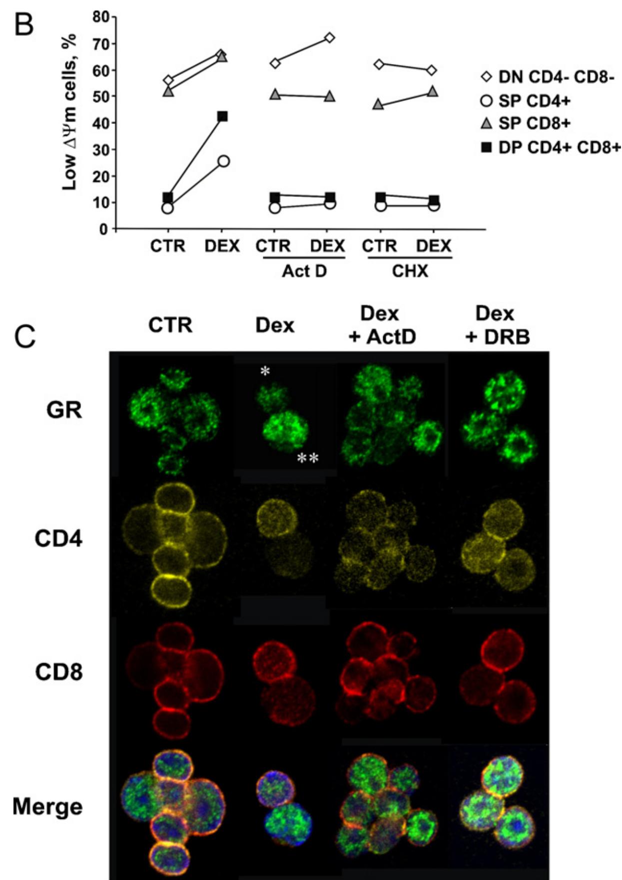

Fig. 4. (*Continued*).

It is well known that glucocorticoid-induced thymocyte apoptosis can be prevented by inhibitors of transcription and translation, such as actinomycin D and cycloheximide, respectively [2,9]. These early observations have led to the easy concept that the initiation of apoptosis in thymocytes requires *de novo* synthesis of components of a suicide program encoded by putative "killer" genes [9]. This concept was reevaluated 10 years ago using a panel of protein synthesis inhibitors comprising cycloheximide, emetine and puromycin [17]. The main outcome of the study was that complete inhibition of protein synthesis is not needed for full protection, and the magnitude of translational arrest induced by these inhibitors does not correlate with their anti-apoptotic effect, suggesting that the effects of translational inhibitors on thymocyte apoptosis are non specific.

Our results confirmed the inhibitory role of two transcription inhibitors Act D and puromycin as well as two translation inhibitors CHX and DRB in Dex-induced loss of $\Delta\Psi_m$ in murine thymocytes. They also provide clear evidence that these drugs disturb GR intra-cytoplasmic signalling in all thymocyte subsets, whether or not sensitive to Dex. These results strikingly resemble the ones observed with geldanamycin, a HSP90 inhibitor, known to inhibit both GR turnover and glucocorticoid-induced apoptosis by interfering with GR macromolecular complexes stability [20,21,36]. GR macromolecular complexes comprise several heat shock proteins (HSP), chaperones and immunophilins [3]. These proteins, and especially HSP90, maintain GR in a conformation suitable for ligand binding, protect the receptor against rapid degradation by the proteasome and are required for ligand-induced nuclear translocation. Our results indicate that Act D and CHX may affect steroid receptor macromolecular complex stability or organization, in a way which can alter glucocorticoid binding, GR activation and degradation (Fig. 2) and apoptotic signalling (Fig. 1). It is unclear at present how these inhibitors may modulate GR trafficking and subsequent apoptosis. However, this depends on the cellular context since the HeLa cell line does not respond as thymocytes and splenocytes do.

It is well-known that the levels of GR are tightly regulated by the proteasome and ligand binding enhances this degradation [33]. Our data show that treatments with CHX or Act D block the degradation of the glucocorticoid receptor (Fig. 2C), suggesting either a labile factor or an inhibitory mechanism in controlling the stability of the receptor within the macromolecular complex. The same effects have already been described for another nuclear receptor, the estrogen receptor, in presence of CHX or Act D [28,31]. This suggests the phenomenon could be directly linked to the stability of several members of the nuclear receptor family. Our data are further supported by another study demonstrating Act D alone may induce a similar accumulation of both GR and p53 proteins in MCF-7 cells [29], suggesting a more common role of these inhibitors on members of this nuclear receptor family.

Alteration of intracellular trafficking by macromolecular synthesis inhibitors has also been reported for estrogen and progesterone receptors [30–32]. Effectively, such inhibitors affect cyclophilins and FK506 binding proteins which are required for steroid receptor activation, interaction with dynein and microtubules and subsequent movement toward the nucleus [37,38] as illustrated by Act D-induced relocation of cyclophilin 40 and FKBP59, the latter to the perinuclear region [32]. In this context, CHX has also been shown as a potent inhibitor of FKBP12 and homologues [39]. Additionally, transcriptional blockade with α-amanitin has been recently associated with p53 translocation to mitochondria and subsequent apoptosis [40]. Thus, macromolecular inhibitors such as Act D, puromycin, CHX and DRB may directly

target components of the GR macromolecular complex, altering GR activation, GR stability, nuclear shuttling and apoptosis induction.

Inhibition of GR intracellular signalling may however explain apoptosis prevention by these drugs independently of the GR activity involved, e.g. transactivation, transrepression or non-genomic events. Thus, macromolecular inhibitors may potentially interfere with cytoplasmic GR signalling pathways and not only nuclear translocation. In accordance, CHX at 2 μM fully inhibited Dex-induced apoptosis (Fig. 1B) without affecting nuclear translocation (Fig. 3B). Interestingly, the DP subset that express the lowest GR level is the most sensitive to Dex treatment, suggesting different pathways may be activated in each subset [13,14]. These effects could be due to non-genomic effects such as caspase activation within the cytoplasm, by GR-interacting proteins such as Src kinase [41], or mitochondrial translocation of the ligand-bound GR [16,42]. It has recently been shown that in the most glucocorticoid-sensitive DP subpopulation, mitochondrial GR translocation correlates with glucocorticoid-induced sensitivity [16]. Moutsatsou [43] and Psarra [44], however, described a mitochondrial GRα localization in various cell lines and organs, including mouse brain and liver while GRβ was primarily restricted to nucleoli. Psarra et al. have found a mitochondrial GR localization in untreated cells where it may have a metabolic activity after binding to specific mitochondrial GRE [45]. In this system, glucocorticoid treatment induced thereafter a time- and dose-dependent GR export from the organelles [46]. Finally, mitochondrial GR localization in the presence or in the absence of Dex as well as alternative non-genomic pathways deserve further investigation to fully decipher GR signalling in thymocytes apoptosis.

As a conclusion, the present study demonstrates the lack of specificity of these macromolecular synthesis inhibitors in thymocyte apoptosis as previously suggested by Chow et al. [17], by finding one clue for it. Act D, DRB, CHX and puromycin interfere with GR trafficking and cannot be considered only as inhibitors of GR-induced transcription and translation. Therefore, the interference of such metabolic inhibitors with nuclear receptors signalling is an intriguing question and may proceed at various levels which remain to be fully clarified. Finally, their role as *de novo* transcriptional and translational inhibitors in apoptosis must be exercised with caution.

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