Protein-synthesis-dependent induction of annexin I by glucocorticoid

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We demonstrate that annexin I/lipocortin I (lipo I) gene expression is regulated by dexamethasone (DEX) in mouse 3T3-L1 fibroblasts and LA-4 lung epithelial cells. We have characterized this induction further in 3T3-L1 fibroblasts. At 24 h after addition of DEX, the levels of lipo I mRNA and protein increased 5-fold and 1.5-fold respectively. Time-course experiments revealed that the induction was delayed by 2–4 h after DEX addition. Half-maximal induction of both lipo I mRNA and protein was achieved with 10 nM-DEX. Both actinomycin D and cycloheximide blocked the DEX effect on lipo I mRNA expression. These results indicate that the induction of lipo I by DEX has a transcriptional component and requires protein synthesis *de novo*.

INTRODUCTION

Annexins are a family of similar Ca²⁺-dependent phospholipid-binding proteins [1]. To provide continuity, the name 'annexin' has been adopted to describe the related members of this family, which include the following proteins: lipocortin I (calpactin II, p35) [2] and lipocortin II (calpactin I, p36) [3,4], calelectrins [5,6], endonexins [7,8], calcimedins [9], chromobindins [10], placental anticoagulant proteins [11,12] and proteins I, II and III [13]. The individual members of the family were independently isolated, but found to be structurally related through a conserved core domain. The core structure is formed from a stretch of 72 amino acids which is repeated in each family member four or eight times [4,14]. Within each repeat, there is a conserved 17-amino-acid consensus region, which is potentially the site for Ca²⁺/phospholipid binding [1]. Even though the annexins share a high degree of sequence similarity within the core domain, their N-terminal ends are unique [14]. In annexin I/lipocortin I and annexin II/lipocortin II the N-terminal sequence contains a tyrosine residue that has been shown to be phosphorylated by the epidermal-growth-factor receptor/kinase and by pp60^{src} respectively [15,16]. Although these proteins have had a variety of different names and been implicated in a number of cellular roles, a well-defined and accepted biological function has not been determined for any of the annexins.

Annexin I/lipocortin I (which for simplicity will be designated lipo I) has been implicated in growth-factor-dependent intracellular signal transduction [17,18]. Lipo I was, however, initially identified as a glucocorticoid-responsive protein in macrophages and neutrophils [19–21]. Cellular responses to glucocorticoids are mediated by three separate mechanisms. The best-documented mechanism is designated a primary response, which occurs very rapidly and is independent of protein synthesis. Examples of genes that are regulated by this mechanism include the metallothionein gene [22] and the mouse mammary-tumour virus DNA [23,24] which are dependent on the direct interaction of the glucocorticoid-receptor complex with regulatory sites, termed 'glucocorticoid response element', in the 5' flanking region of the metallothionein gene or the viral long terminal repeat.

Secondary responses to glucocorticoids all require protein synthesis *de novo*, but can be further divided into two dis-

tinguishable classes [25]. Rapid secondary responses resemble the primary response by displaying little or no lag time after glucocorticoid administration. The rat α_1 -acid glycoprotein gene is an example of this response, requiring protein synthesis *de novo* and showing an induction by dexamethasone during the first 1 h of treatment [26]. Delayed secondary responses, on the other hand, exhibit a lag time of several hours between the addition of steroids and the initiation of the glucocorticoid-induced mRNA accumulation. This type of response is best exemplified by the rat α_{2u} -globulin gene [27].

Which of the above mechanisms regulates lipo I expression has not been investigated. Wallner *et al.* [2] showed an increase in lipo I mRNA in rat peritoneal macrophages within 2 h of administering dexamethasone (DEX), with no evidence for a change in protein levels. Using a mouse lipo I cDNA isolated from a mouse spleen cDNA library as a probe to examine glucocorticoid-dependent lipo I gene expression, we were unable to reproduce the DEX-dependent induction of lipo I in peritoneal macrophages as reported by Wallner *et al.* [2]. However, we show here that lipo I gene expression is in fact inducible by glucocorticoids in two mouse cell lines: 3T3-L1 fibroblasts and LA-4 lung epithelial cells. We demonstrate for the first time that DEX induces both lipo I mRNA and protein. We also present evidence which illustrates that the induction of lipo I mRNA exhibits a lag time of 2–4 h, is regulated at the transcriptional level, and is dependent on protein synthesis *de novo*. Therefore, regulation of lipo I expression by glucocorticoids is mediated by a delayed secondary response. Given the expanding number of genes which exhibit this delayed protein-synthesis-dependent steroid response, it is likely that these genes will play an important role in our understanding of tissue-specific glucocorticoid regulation.

EXPERIMENTAL

DEX experiments in animals

Balb/c mice (10–12 weeks old) were injected intraperitoneally with 125 μ g of DEX phosphate (Quad Pharmaceuticals, Indianapolis, IN, U.S.A.)/kg for various periods of time. Peritoneal macrophages were collected by washing the peritoneal cavity with 5 ml of phosphate-buffered saline (120 mM-NaCl/

Abbreviations used: lipo I, lipocortin I; DEX, dexamethasone; DMEM, Dulbecco's modified Eagle's medium.

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2.7 mM-KCl/10.0 mM-phosphate buffer, pH 7.4). Cells from three to five animals [$(1-1.5) \times 10^6$ cells per mouse] were pooled, centrifuged at 1000 *g* for 3 min, and lysed for RNA isolation. In experiments with primary peritoneal macrophages, cells from mice were collected by washing the peritoneal cavity with 5 ml of phosphate-buffered saline, followed by centrifugation for 3 min at 1000 *g*. The cells were resuspended at a density of 5×10^6 cells/10 ml in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with $1 \times$ antibiotic antimycotic solution (Sigma) and 4 mM-L-glutamine (Sigma), plated out on a 10 cm tissue-culture plate, and allowed to adhere to the plate for 20 h. Cells were washed three times with DMEM supplemented with 4 mM-L-glutamine and $1 \times$ antibiotic antimycotic solution, treated with 1 μ M-DEX, and lysed for RNA isolation.

Cell culture

Mouse lung epithelial cell line LA-4 was cultured in 10 cm tissue-culture plates at 37 °C, under 5% CO₂ in air, in DMEM supplemented with 10% (v/v) fetal-bovine serum (Flow Laboratories), $1 \times$ antibiotic antimycotic solution and 4 mM-L-glutamine. 3T3-L1 cells were cultured in 10 cm tissue-culture plates at 37 °C, under 7% CO₂ in air, in DMEM supplemented with 10% calf serum (Flow Laboratories), $1 \times$ antibiotic antimycotic solution and 4 mM-L-glutamine. Generally, 2×10^5 cells were plated and then fed every 2 days until they reached confluence. At confluence, cells were fed with fresh medium and then cultured for 2 additional days before they were used in the various experiments described below.

DEX experiments

Before treatment with hormones and/or inhibitors, cell culture medium was changed from DMEM with 10% calf serum to DMEM with 1% fetal bovine serum (Flow Laboratories) unless otherwise indicated. DEX (Sigma) in ethanol was then added to cell culture medium to final concentrations of 1 μ M-DEX and 0.4% ethanol. Accordingly, control plates were treated with an equivalent volume of ethanol. In metabolic inhibition experiments, either 4 μ g of actinomycin D (Sigma)/ml or 20 μ M-cycloheximide (Sigma) was added to the culture medium.

RNA isolation

RNA was isolated by lysing cells in a guanidinium thiocyanate/sarcosyl solution, followed by acid phenol/chloroform extraction and ethanol precipitation as described previously [28]. Typically, 3.0×10^6 cells from a 10 cm plate were lysed with 3 ml of guanidine thiocyanate solution, containing 4 M-guanidine thiocyanate, 25 mM-sodium citrate (pH 7.0), 0.5% sarcosyl and 100 mM-2-mercaptoethanol. Then 300 μ l of 2 M-sodium acetate (pH 4.0), 3 ml of water-saturated phenol and 1 ml of chloroform/3-methylbutan-1-ol (24:1, v/v) were added in stepwise fashion. The mixture was centrifuged at 10000 *g* for 10 min to separate phases. The aqueous phase was mixed with 3 ml of propan-2-ol, and total RNA was precipitated by incubating the mixture at -20 °C for at least 1 h, followed by centrifugation at 10000 *g* for 30 min. The RNA pellet was then resuspended in 600 μ l of guanidine thiocyanate solution and concentrated by precipitation with propan-2-ol and ethanol.

Northern analysis

A 20 μ g portion of total RNA was loaded on a 1% Mops/formaldehyde/agarose gel as described previously [29]. The gels were run for 12-16 h at 40 V with constant buffer recirculation and then subjected to three 30 min washes with water, one 45 min wash with 0.05 M-NaOH and 0.01 M-NaCl, one 45 min wash with 0.1 M-Tris/HCl (pH 8.0), and a 30 min wash with 0.089 M-Tris/borate/0.089 M-boric acid/2 mM-EDTA

(pH 8.0). The RNA was electrophoretically transferred from the gel to a non-charged nylon membrane (Genescreen; NEN) and cross-linked with u.v. light [30].

Probe preparation and Northern hybridization of RNA blots

Mouse lipo I probes were synthesized with [α -³²P]dATP (NEN; 800 μ Ci/mmol) either by random primer extension with hexamer primers (Pharmacia) [31] using a 906 bp mouse lipo I cDNA insert, or by an M13 single-stranded probe synthesis method [30] using an M13mp18 clone containing the same partial mouse lipo I cDNA in the appropriate orientation. A mouse β -actin cDNA insert was obtained from Dr. Lawrence Kedes (Stanford University), from which probes were synthesized by random primer extension. Oligonucleotide probes derived from rat metallothionein I and II cDNA sequences were made by kinase reaction with [γ -³²P]ATP (ICN; 7000 μ Ci/mmol) as described [29]. Hybridization was performed for 12-16 h in a buffer containing 1% BSA (Sigma, A-7511), 1 mM-EDTA, 0.5 M-Na₂HPO₄ (pH 7.2) and 7% SDS at 60 °C. The blots were then subjected to three 10 min washes in 1 mM-EDTA/40 mM-Na₂HPO₄ (pH 7.2)/1% SDS at 65 °C and exposed to Kodak X-OMAT AR film for various periods of time. Relative intensity of each sample on the autoradiograph was quantified with the Bio Image Visage 110 imaging system (Millipore Bio Image, Ann Arbor, MI, U.S.A.).

Fractionation of lipo I protein and Western analysis

3T3-L1 fibroblasts were grown to confluence in 35 mm culture dishes in DMEM containing 10% calf serum. Then 2-day post-confluent cells were treated with the appropriate concentration of DEX (dissolved in 95% ethanol) or by an equal volume of ethanol in DMEM containing 1% fetal-bovine serum. After the designated time, the cells were washed with 3×3 ml of phosphate-buffered saline (pH 7.4) and then homogenized under three different conditions. Specifically, cells were extracted in 0.5 ml of buffer, composed of 20 mM-Tris (pH 7.3), 0.25 M-sucrose and 5 μ g of aprotinin (Sigma)/ml, alone or containing 1 mM-EDTA or 1 μ M-CaCl₂. The lysed cells were then centrifuged at 212000 *g* for 70 min to collect the soluble (cytosolic) and total membrane fraction. The membrane pellet was resuspended in the Tris/sucrose buffer, alone. A sample of each fraction (representing 5×10^4 cells) was applied to a SDS/10%-acrylamide gel for separation of proteins under reducing conditions [32]. After electrophoresis, the gel was equilibrated for 1 h in buffer (25 mM-Tris base, pH 8.3; 192 mM-glycine; 20% methanol) and then electrotransferred to nitrocellulose. The nitrocellulose was stained with Amido Black and then blocked in buffer (20 mM-Tris base, pH 7.4; 0.5 M-NaCl) containing 3% BSA for 3 h. Antibody to lipo I (kindly provided by Dr. R. Blake Pepinsky) was added at 1:1000 dilution to blocking buffer and incubated with the nitrocellulose overnight at 4 °C. The nitrocellulose was next washed in buffer containing 1% BSA for 1 h, then incubated with iodinated Protein A in blocking buffer for 1 h, and finally washed three times in blocking buffer over 1 h. The blot was air-dried and exposed to Kodak X-OMAT AR film. Lipo I was quantified for comparative purposes by video densitometry using the Bio Image Visage 110 system.

RESULTS

Effect of DEX on lipo I expression in primary peritoneal macrophages and cell lines

It has been reported that annexin I/lipocortin I (lipo I) mRNA is induced in peritoneal macrophages after a 2 h exposure to DEX [2]. In an effort to reproduce these results, we injected animals intraperitoneally with DEX, and then isolated peri-

Fig. 1. Effect of DEX on lipo I mRNA level in mouse peritoneal macrophages and mouse cell lines, examined by Northern analysis

Total RNA isolated from cells (20 µg) was examined as described in the Experimental section. The membrane was re-probed with a mouse actin cDNA, which served as an internal control. The intensity of the corresponding lipo I and actin signals was quantified by video densitometry. (a) Total RNA isolated from peritoneal macrophages of mice injected with 125 µg of DEX/kg for the time indicated (lanes 1 and 2) and from primary peritoneal macrophages treated with 1 µM-DEX for the time indicated (lanes 3 and 4). The Northern blot was also probed with oligonucleotide probes derived from the rat metallothionein (MT) cDNA sequence, which served as a positive control for DEX-responsiveness. (b) Total RNA isolated from mouse 3T3-L1 fibroblasts (lanes 1 and 2) and mouse lung epithelial cell line LA-4 (lanes 3 and 4). Lanes 1 and 3 are total RNA from control cells, and lanes 2 and 4 are from cells treated with 1 µM-DEX for 24 h.

toneal macrophages. Unlike the previous report, we detected no significant change in lipo I mRNA level in peritoneal macrophages after 2 h of DEX treatment (Fig. 1a, lane 2) as compared with peritoneal macrophages isolated from control animals (Fig. 1a, lane 1). As an internal control for DEX activity, the blot was washed and then re-probed with another DEX-responsive gene, that for metallothionein. Unlike that for lipo I, the metallothionein mRNA increased 15-fold in peritoneal macrophages after 2 h of DEX treatment. Furthermore, treatment of primary peritoneal macrophages, which were first allowed to adhere to tissue-culture plates for 20 h, with 1 µM-DEX for 20 h also resulted in no steroid-dependent change in lipo I mRNA level (Fig. 1a, lanes 3 and 4). In contrast, 20 h-DEX-treated primary peritoneal macrophages showed a 5-fold increase in metallothionein mRNA.

Given that the lipo I gene was not responsive to DEX in peritoneal macrophages, we examined other cell types for DEX inducibility. As shown in Fig. 1(b), we found that 1 µM-DEX caused a reproducible induction of lipo I mRNA in either 3T3-L1 mouse fibroblasts or LA-4 mouse lung epithelial cells, after a 24 h treatment.

Fig. 2. Effect of DEX on lipo I mRNA level in 3T3-L1 fibroblasts, examined by Northern analysis

(a) Total RNA (20 µg per lane) isolated from a time course of DEX-treated cells maintained in DMEM with 1% fetal-bovine serum was electrophoretically fractionated on a 1% Mops/formaldehyde/agarose gel, blotted on to a nylon membrane, and probed with a ³²P-labelled mouse lipo I cDNA as described in the Experimental section. The membrane was re-probed with a mouse actin cDNA, which served as an internal control, and with oligonucleotide probes derived from rat metallothionein (MT) cDNA sequence, which served as a positive control for DEX-responsiveness. Lanes 1, 2, 4, 6, 8, 10 and 13 correspond to total RNA isolated from control cells at 0, 2, 4, 6, 8, 10, 12 and 24 h respectively. Lanes 3, 5, 7, 9, 11 and 14 are total RNA isolated from cells treated with 1 µM-DEX for 2, 4, 6, 8, 12 and 24 h respectively. (b) A graphical summary of eight independent DEX experiments (○, control; ●, DEX). The intensity of the corresponding lipo I and actin signals was quantified by video densitometry. Data are means ± S.D. The lipo I signal (E_l) in each lane was derived by using actin as an internal control. From eqn. (1), individual lane variation was adjusted by plotting the ratio of lipo I intensity at a specific time (*t*) to lipo I intensity at zero time (0) divided by the ratio of actin intensity at *t* to the actin intensity at 0:

$$E_l = \frac{\text{lipo } I_t / \text{actin}_t}{\text{lipo } I_0 / \text{actin}_0} \quad (1)$$

Induction of lipo I mRNA by DEX in 3T3-L1 cells

To evaluate the effects of DEX on lipo I gene expression, we treated confluent 3T3-L1 fibroblasts with 1 µM-DEX for various times. We isolated total RNA from control and steroid-treated cells. The results in Fig. 2(a) demonstrate the time-dependent induction of lipo I mRNA in response to DEX treatment. As compared with controls, an induction was observed in treated cells as early as 4 h, with the largest increase at 24 h. A quantitative summary of densitometric data from eight independent experiments is shown in Fig. 2(b). At 24 h of DEX exposure, the level of lipo I mRNA in treated cells was on average 5-fold higher than in control cells; that in the control cells was essentially constant over the 24 h period of incubation. These experiments also demonstrate that there was an initial lag

Fig. 3. Effect of different concentrations of DEX on lipo I mRNA level

A graphical summary is shown of five independent dosage experiments, quantified as described in Fig. 2. Fibroblasts were treated with various concentrations of DEX for 12 h, followed by Northern analysis as described in the Experimental section. Data are means $\pm$ S.D.

Fig. 4. Fractionation of lipo I in 3T3-L1 fibroblasts

Confluent fibroblasts were treated for 24 h in the presence (lanes 4, 5 and 6) or absence (lanes 1, 2 and 3) of 1 μ M-DEX. Because DEX was dissolved in 95% ethanol, control cells contained an equivalent amount of the solvent. At the appropriate time, cells were extracted in Tris/sucrose buffer in the presence of 1 mM-EDTA (lanes 1 and 4) or 1 μ M-CaCl₂ (lanes 3 and 6), or in the absence of both (lanes 2 and 5). Cells were homogenized, and the cytoplasmic fraction was separated from the membrane fraction by centrifugation as described in the Experimental section. Lipo I was identified in each fraction by immunoblotting as described in the Experimental section. Shown is the autoradiograph of a representative experiment. The presence of lipo I in the membrane and in the cytosolic fractions is as indicated. The bands were quantified by image analysis. In this experiment, a 2.45-fold induction by dexamethasone was observed.

before an increase in lipo I mRNA was apparent. In contrast, there was no delay in the induction of metallothionein-gene expression by DEX. As an additional control, we repeated the experiments with water-soluble DEX phosphate and obtained results (not shown) identical with those of the experiments with ethanol-soluble DEX.

DEX dosage response

To determine whether the DEX-dependent lipo I mRNA induction occurs at physiological concentrations of glucocorticoid, we incubated cells for 12 h with increasing concentrations of DEX (ranging from 0.1 nM to 10 μ M). The results of these experiments are summarized in Fig. 3. Fig. 3 shows that half-maximal induction of lipo I mRNA by DEX occurred at 10 nM. This concentration is lower than the physiological ranges

Fig. 5. Effect of DEX on lipo I protein level

Confluent 3T3-L1 fibroblasts were treated for various times in the presence of 1 μ M-DEX. Control plates were assayed at equivalent times in the presence of an equal volume of the solvent. After extraction with Tris/sucrose buffer containing 1 mM-EDTA, proteins in the soluble fraction of the cell lysate were separated by SDS/PAGE, and transferred to nitrocellulose. Lipo I was identified by Western analysis as described in the Experimental section. The bands were then quantified by video densitometry. The data represent four independent experiments, and are means $\pm$ S.D. Inset: fibroblasts were treated for 24 h in the presence of various concentrations of DEX or an equivalent volume of ethanol. The data represent a single experiment.

(140 nM–1.1 μ M) of cortisol [33]. This increased sensitivity to the hormone may be accounted for by the greater affinity of the glucocorticoid receptor for this synthetic glucocorticoid [34].

Effect of DEX on lipo I protein level

Given the results presented in Figs. 2(a) and 2(b) on mRNA level, we then evaluated the effects of DEX on lipo I protein level. Because it has been reported that lipo I can be fractionated into either the cytoplasm or plasma membranes, depending on the intracellular Ca²⁺ levels [17], we first determined the subcellular location of lipo I under various conditions. As demonstrated in Fig. 4, the presence of Ca²⁺ caused lipo I to co-localize with the membrane fraction. In the presence of EDTA, lipo I was found exclusively in the cytosol. Fig. 4 also shows that DEX caused an increase in lipo I protein in treated 3T3-L1 cells as compared with control cells. We further characterized this induction, as illustrated in Fig. 5 (only densitometric analysis is presented). Averaging data from four independent experiments, we show that lipo I increased by 1.5-fold. The concentration-dependence was similar to mRNA as shown in the inset in Fig. 5.

Effect of actinomycin D on the DEX induction

The DEX-dependent increase in lipo I expression that we observed could be a consequence of a direct increase in transcriptional rate, the prolongation of lipo I mRNA half-life, or both. To address the underlying mechanism for the increase in lipo I mRNA level, we repeated the glucocorticoid experiments as described above in the presence of 4 μ g of actinomycin D/ml to block RNA synthesis *de novo*. As shown in Fig. 6(a), we found no effect of actinomycin D on expression of lipo I mRNA in controls within the time period of this experiment, indicating that lipo mRNA half-life is greater than 12 h. In contrast, the induction by DEX was prevented by the inhibitor, which is graphically represented in Fig. 6(b). The results suggest that the mechanism of DEX induction of lipo I expression is dependent on RNA synthesis *de novo*.

Fig. 6. Effect of actinomycin D on the DEX-dependent induction of lipo I mRNA

(a) Total RNA was isolated from 3T3-L1 fibroblasts treated with 1 μ M-DEX, 4 μ g of actinomycin D (ACT)/ml, or simultaneously treated with both DEX and actinomycin D, and examined by Northern analysis as described in the Experimental section. Lane 1 corresponds to total RNA isolated from 3T3-L1 fibroblasts before treatment. Lanes 2, 6 and 10 are from control cells maintained for the designated times. The remaining lanes were from cells treated with the steroid (lanes 3, 7 and 11), inhibitor alone (lanes 4, 8 and 12) or co-treated with both compounds (lanes 5, 9 and 13) for 4, 8 and 12 h respectively. (b) A graphical summary of the actinomycin D (ACT) experiment, quantified as described in Fig. 2: $\circ$, control; $\bullet$, DEX; $\triangle$, ACT; $\blacktriangle$, ACT+DEX.

Fig. 7. Effect of cycloheximide on the DEX-dependent induction of lipo I mRNA

(a) Total RNA was isolated from 3T3-L1 fibroblasts treated with 1 μ M-DEX, 20 μ M-cycloheximide (CHX) or simultaneously treated with both DEX and cycloheximide and examined by Northern analysis as described in the Experimental section. Lane 1 corresponds to total RNA isolated from 3T3-L1 fibroblasts before treatment. Lanes 2, 6 and 10 are from control cells maintained for the designated times. The remaining lanes were cells treated with the steroid (lanes 3, 7 and 11) or inhibitor alone (lanes 4, 8 and 12) or co-treated with both compounds (lanes 5, 9 and 13) for 4, 8 and 12 h respectively. (b) A graphical summary of the cycloheximide experiment, quantified as described in Fig. 2: $\circ$, control; $\bullet$, DEX; $\triangle$, CHX; $\blacktriangle$, CHX+DEX.

Effect of cycloheximide on the DEX response

The effect of DEX on lipo I expression in 3T3-L1 cells was first detectable between 2 and 4 h after addition of DEX (see Figs. 2a and 2b). The lag period of 2 h provides more than adequate time for protein synthesis *de novo*, which may be a prerequisite for the DEX response. To determine whether protein synthesis *de novo* might play a role in the induction of lipo I mRNA by DEX, we repeated the glucocorticoid treatments as described above, with concurrent addition of 20 μ M-cycloheximide. As shown in Figs. 7(a) and 7(b), the normal induction over 12 h was observed, whereas cells exposed to cycloheximide alone or cells treated with both DEX and cycloheximide show no detectable difference in the lipo I mRNA level as compared with control cells. These results strongly imply that protein synthesis *de novo* is required for the DEX-dependent induction of lipo I mRNA. In contrast with that for lipo I, the DEX-dependent increase in metallothionein mRNA was not blocked by treatment with cycloheximide (Fig. 7a), but instead showed a 'superinduction'.

To demonstrate further the importance of new protein synthesis, cycloheximide was added to confluent cells 4 h after addition of DEX, thus allowing time for the development of the induction process before addition of the inhibitor. As shown in Fig. 8, the increase in lipo I mRNA in cells treated with DEX

Fig. 8. Effect of cycloheximide on the DEX-dependent induction of lipo I mRNA in 3T3-L1 fibroblasts pre-treated with DEX for 4 h

Total RNA was isolated and analysed from cells pre-treated for 4 h with 1 μ M-DEX, followed by additional 8 h exposure to 20 μ M-cycloheximide (CHX) as described in the Experimental section. Lanes show total RNA isolated from: 1, control cells; 2, cells treated with DEX for 12 h; 3, cells which were transferred to DMEM with 1% fetal-bovine serum for 4 h and then treated with cycloheximide for an additional 8 h; 4, cells treated with DEX for 4 h, followed by exposure to cycloheximide for 8 h.

alone (lane 2) or with both DEX and cycloheximide (lane 4) is identical when compared with control cells (lanes 1 and 3). This experiment illustrates that the presence of DEX for 4 h before inhibition of protein synthesis by cycloheximide is sufficient to allow the induction process to develop.

DISCUSSION

Glucocorticoids induce a variety of biological responses in mammalian cells [34]. Lipo I was initially identified by its sensitivity to glucocorticoids [19–21,35]. Wallner *et al.* [2] have reported that, 2 h after peritoneal injection of DEX, lipo I mRNA level in rat peritoneal macrophages increased by 6-fold. However, several recent studies using peripheral lymphocytes [36], primary macrophages [36,37], lymphocyte cell lines [38,39] and umbilical-cord endothelial cells [40] have reported the inability to detect any increase in lipo I mRNA or protein after DEX treatment. Because there were differences in the protocols of those studies compared with Wallner *et al.* [2], our initial goal in evaluating the DEX effect on lipo I expression was to reproduce faithfully the conditions *in vivo* used by Wallner *et al.* [2]. However, after a large number of experiments, we, too, were unable to observe any increase in lipo I mRNA in peritoneal macrophages after DEX treatment (Fig. 1a, lanes 1 and 2). We further examined the effect of DEX after treatment of primary peritoneal macrophages *in vitro*, and we again detected no change in the cellular level of lipo I mRNA after 20 h of DEX treatment (Fig. 1a, lanes 3 and 4), consistent with the study by Northup *et al.* [37]. However, when the same membranes were re-probed with a metallothionein probe, we were able to show clearly that DEX induced metallothionein expression both *in vivo* and in primary peritoneal macrophages (Fig. 1a).

In contrast with peritoneal macrophages, we have observed that DEX induces lipo I expression in three mouse cell lines: 3T3-L1 fibroblasts, LA-4 lung epithelial cells (Fig. 1b), and HC11 mammary epithelial cells (K. Horlick & H. S. Nick, unpublished work). In 3T3-L1 cells, we have further demonstrated that both lipo I mRNA and protein can be induced by DEX with equal sensitivity, which represents the only evidence that DEX induces both lipo I mRNA and protein expression. Interestingly, there was a difference in fold response of mRNA and protein. At 24 h after addition of 1 μ M-DEX, the lipo I mRNA increased by 5-fold (Fig. 2b), whereas lipo I protein increased by 1.5-fold (Fig. 5). Additional experiments revealed that DEX decreased overall cellular protein synthesis by as much as 50% within 24 h (M. Gianjianpour & S. C. Frost, unpublished work), suggesting that the potential DEX-dependent increase in lipo I protein was probably 3–5-fold that in the mRNA.

Consistent with our observations, Phillips *et al.* [41] have reported that fluocinolone acetonide, a synthetic glucocorticoid, is able to induce lipo I mRNA in Swiss 3T3 cells. However, they reported that both serum addition and confluent cells were a prerequisite for DEX-dependent induction of lipo I expression. Although we obtained similar DEX induction of lipo I expression, the response in 3T3-L1 cells was independent of serum, and identical induction was observed whether confluent or exponentially growing cells were employed (W. T. Wong, unpublished work). The ability to induce lipo I with DEX in specific cell lines, such as 3T3-L1, HC11, LA-4 and Swiss 3T3 fibroblasts, as compared with the negative results of other investigators [36–40], may arise because these fibroblasts and epithelial cells express specific factors which are required for DEX-dependent lipo I expression, as discussed below.

On the basis of our observations with actinomycin D (Figs. 6a and 6b), the major effect of DEX on lipo I mRNA level most likely occurs at the transcriptional level. We have also found that

inhibition of protein synthesis by cycloheximide abolishes the induction of lipo I mRNA by DEX (Figs. 7a and 7b). These results are different from the well-characterized glucocorticoid-dependent induction of mouse mammary-tumour virus DNA and the metallothionein gene, in which cycloheximide does not block the induction [22,23]. The primary glucocorticoid response is also characterized by rapid induction. In mouse mammary-tumour virus DNA, for instance, the initiation of the steroid-induced mRNA accumulation can be detected within 1 h of DEX addition [24]. In contrast, our DEX response is delayed by 2 h or more (Fig. 2a). Maximum induction of lipo I mRNA by DEX requires at least 12 h of hormone treatment. Together, the unusually slow DEX-response kinetics and the ability of cycloheximide to abolish the DEX effect on lipo I expression suggest that protein synthesis *de novo* is required for DEX action. In addition, the fact that cycloheximide can no longer inhibit the DEX response if added 4 h after cells have been treated with DEX (Fig. 8) implies that the newly synthesized protein factors required for DEX-dependent induction have a half-life greater than 8 h.

A number of DEX-inducible genes (including those for phosphoenolpyruvate carboxykinase [42], α_1 -acid glycoprotein [26], albumin [43], tryptophan 2,3-dioxygenases [44], α_{2u} -globulin [25,27] and urea-cycle enzymes [45]) have been reported to require protein synthesis for DEX induction. This type of steroid response is designated as secondary glucocorticoid response, to distinguish it from the primary response, which is independent of protein synthesis. Two types of secondary response have been described. Rapid secondary response shows little or no lag time after glucocorticoid addition. An example of this type of response is the rat α_1 -acid glycoprotein gene, which requires ongoing protein synthesis but shows an induction by dexamethasone during the 1 h of treatment [26]. In contrast with the rapid secondary response, the delayed secondary response, such as that of α_{2u} -globulin gene, shows a lag time of several hours between the administration of steroids and the appearance of induced mRNA [27]. Since the induction of lipo I mRNA by DEX displays a lag time of at least 2 h, lipo I gene is most likely regulated by a delayed secondary response.

The mechanism for the secondary response in the genes discussed above, and specifically that of lipo I, is unknown. Hypothetically, this may be explained via the induction by DEX of an additional gene product, which directly causes the stimulation of lipo I gene expression. Alternatively, DEX may cause the induction of proteins which function in concert with the glucocorticoid receptor in activating lipo I transcription. Recently, Hess *et al.* [25] have reported that purified glucocorticoid receptors bind selectively *in vitro* to a specific DNA sequence, termed a delayed secondary glucocorticoid response element, in the promoter region of the α_{2u} -globulin gene. These investigators have suggested that this regulatory sequence might be important for mediation of the delayed DEX response, thus implying that newly synthesized proteins, in conjunction with the glucocorticoid receptor, orchestrate this response. We have recently cloned the mouse lipo I structural gene (K. Horlick, unpublished work), and an analysis of the 5' flanking region has revealed sequences which are similar to the delayed secondary glucocorticoid response elements defined in the α_{2u} -globulin gene [46]. It should be investigated whether these potential regulatory sequences mediate the DEX-dependent response of the lipo I gene *in vivo*.

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