Glucocorticoids modulate mRNA levels for G-protein β -subunits

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Adrenalectomy decreases, whereas glucocorticoid treatment increases, the steady-state levels of G-protein β -subunits (G_β) in rat fat-cells. A DNA-excess solution-hybridization assay was established to define the steady-state mRNA levels for G_β [5.8 ± 0.4 amol/ μ g of RNA ($n = 5$) in control fat-cells]. G_β mRNA levels decrease by 20% after adrenalectomy; dexamethasone treatment reverses the decline. Dexamethasone treatment itself increases G_β mRNA levels by 50%.

INTRODUCTION

G-proteins are a family of guanine-nucleotide-binding regulatory proteins involved in transmembrane signalling (Gilman, 1987). G-proteins are heterotrimeric proteins, each composed of an α -subunit in association with a complex of β - and γ -subunits. The α -subunits are unique to each G-protein and provide a basis for classification. G-proteins modulate different effector units such as adenylate cyclase (Gilman, 1987), cyclic GMP phosphodiesterase (Stryer & Bourne, 1987), phospholipase C (Cockcroft, 1987) and ion channels (Neer & Clapham, 1988). Although the heterogeneity of α -subunits could be assumed to dictate single-effector-unit recognition, G-proteins such as G_s have been shown to be capable of regulating multiple effector units (Yatani *et al.*, 1988).

β -subunits of G-proteins (G_β) are highly homologous, migrating as a pair of polypeptides with M_r 35000 and 36000 on SDS/polyacrylamide-gel electrophoresis (Sterweis & Robishaw, 1984; Evans *et al.*, 1986). Two different mRNAs, one encoding $G_{\beta 1}$ (M_r 36000), the other encoding $G_{\beta 2}$ (M_r 35000), have been isolated (Codina *et al.*, 1986; Fong *et al.*, 1987). The predicted amino acid sequences of these subunits share about 90% identity. The conservation of these two similar proteins argues in favour of different functional roles for each. The functional distinctions between the β -subunits, however, remain obscure. An inhibitory role for β -subunits in the adenylate cyclase system has been proposed (Gilman, 1984). According to one model, the complex of $\beta\gamma$ -subunits acts by mass action to remove free GTP-bound $G_{s\alpha}$, thereby decreasing the stimulatory effect of $G_{s\alpha}$ on adenylate cyclase. Direct inhibition of the catalytic subunit of the adenylate cyclase by $\beta\gamma$ -complex has also been reported (Katada *et al.*, 1986). In addition, a stimulatory role for $\beta\gamma$ -complex in the opening of K^+ channels in cardiac tissue has been reported (Logothetis *et al.*, 1987).

G-proteins play a key role in transmembrane signalling, and alteration of the steady-state levels of their subunits or of the equilibria that are believed to exist between α -subunits and $\beta\gamma$ -complexes in the cell may change the overall responsiveness of transmembrane signalling. Hormonal status *in vivo* has been shown to

influence the steady-state levels of various G-proteins. In adipose tissue, hypothyroidism has been shown to be associated with an increase in the steady-state levels of $G_{i\alpha}$, $G_{o\alpha}$ and G_β (Malbon *et al.*, 1985; Milligan *et al.*, 1987; Ros *et al.*, 1988). Thyroid status has also been reported to regulate the steady-state level of $G_{s\alpha}$ in reticulocytes (Stiles *et al.*, 1981). Glucocorticoids, like thyroid hormones, have been shown to modulate the steady-state levels of G-protein subunits. In liver, adrenalectomy decreases the amount of $G_{s\alpha}$ while increasing the steady-state level of $G_{i\alpha}$ (García-Sáinz *et al.*, 1989). Chronic exposure to xenobiotics such as ethanol has been shown to cause heterologous desensitization, decreasing the level of $G_{s\alpha}$ and its mRNA in NG108-15 cells (Mochly-Rossen *et al.*, 1988). The hormonal responses mediated by G-protein-linked receptors have been shown to be modulated in each of these situations. More recently, adrenalectomy was shown to result in a decrease in the steady-state level of G_β in rat fat-cells (Ros *et al.*, 1989). This change appears to contribute to the altered hormonal responsiveness of these cells. In the present work we describe for the first time glucocorticoid regulation of a G-protein subunit, G_β , at the level of mRNA.

EXPERIMENTAL

Construction of the $G_{\beta 1}$ cDNA probe

Based on the sequence of human liver $G_{\beta 1}$, a probe (135 bases) was constructed by the strategy of Linnenbach *et al.* (1986). Synthetic oligonucleotides corresponding to both strands of human liver $G_{\beta 1}$ subunit were synthesized. The 5'-ends of each of the oligonucleotides were phosphorylated by using T4 polynucleotide kinase and [γ - 32 P]ATP as described by Maniatis *et al.* (1982). The single-stranded oligonucleotides were subjected to electrophoresis on 20%-polyacrylamide denaturing gels and the DNA bands, identified by autoradiography, were excised and eluted from the gel. The oligonucleotides were collected by ethanol precipitation, resuspended in 15 μ l of 50 mM-Tris (pH 7.8)/10 mM-MgCl₂ containing 1 mg of spermidine/ml, heated to 75 °C, and annealed by slow cooling to room temperature overnight. The

Abbreviations used: G_β , β -subunit of G-protein (similarly G_α); G_s , G_i , stimulatory and inhibitory G-proteins respectively.

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hybrids, corresponding to nucleotides 270–342 (–10 to +60 with respect to the initiator codon) of the human liver $G_{\beta 1}$, were ligated by using T4 DNA ligase at 4 °C for 24 h. The reaction mixtures were subjected to electrophoresis on non-denaturing 12 % polyacrylamide gels. DNA-containing regions of the gel were identified by autoradiography, excised, and the DNA was eluted from the gel. The DNA was collected by ethanol precipitation. The double-stranded fragment was subcloned into bacteriophage M13 mp18 by using *EcoRI* and *AccI* restriction sites. By subcloning in this fashion, primer extension with labelled nucleotides produces uniformly labelled anti-sense DNA to which G_{β} -subunit mRNA can hybridize. The construction of the probe was verified by sequence analysis by the dideoxy chain-termination method of Sanger *et al.* (1977).

Construction of the G_{β} probe

A 57-base-pair *EcoRI*/*HinP1* fragment corresponding to nucleotides 621–678 (amino acid residues 147–165) of the rat olfactory G_{β} cDNA (Jones & Reed, 1987) was subcloned into bacteriophage M13 mp18 as described by Maniatis *et al.* (1982).

RNA isolation

Total cellular RNA was extracted by guanidine isothiocyanate as described by Chirgwin *et al.* (1979). Formaldehyde/agarose-gel electrophoresis was used to assess the integrity of the RNA. The yield of total cellular RNA did not vary significantly among the treated and control animals.

Solution-hybridization-probe synthesis

The probe was synthesized by primer extension of the single-stranded M13 template DNA with [α - 32 P]dCTP (220 Ci/mmol) by the method of Williams *et al.* (1985), as modified by Hadcock & Malbon (1988a).

Solution-hybridization assay

Uniformly radiolabelled probe (100 pg; 7700 Ci/mmol) was incubated with increasing amounts of M13 DNA template or total cellular RNA (50 μ g) for 60 h at 68 °C. The amount of G-protein mRNA in each sample was determined as described by Hadcock & Malbon (1988a). The resistance of the probe alone to digestion by S1 endonuclease was less than 1 %.

RNA-blot analysis

A 50 μ g portion of total cellular RNA was electrophoresed and transferred to nylon membranes as described by Shackelford & Varmus (1987). The blots were pre-hybridized for 2 h and then probed for 24–36 h with the uniformly labelled probe [(1–2) $\times 10^6$ c.p.m./ml], as described by Maniatis *et al.* (1982). The blots were washed twice with 2 $\times$ SSC [SSC = 15 mM-sodium citrate (pH 7.0)/150 mM-NaCl]/0.1 % SDS for 10 min at room temperature and four times (15 min each) with 0.2 $\times$ SSC/0.1 % SDS at 55 °C. The blots were then subjected to autoradiography.

RESULTS AND DISCUSSION

Glucocorticoids regulate the expression of G_{β} subunits (Ros *et al.*, 1989). Previous studies from this laboratory have shown that adrenalectomy is associated with a decrease (> 50 %) in the steady-state levels of G_{β} subunits

Fig. 1. RNA-blot analysis of G_{β} subunits: effect of glucocorticoid status

Total cellular RNA (50 μ g) of adipose tissue isolated from control (C, lanes 1 and 2), adrenalectomized (A, lane 3), adrenalectomized dexamethasone-treated (AD, lane 4) and control dexamethasone-treated (CD, lane 5) rats was subjected to 1.2 % agarose/formaldehyde-gel electrophoresis and transferred to nylon membranes. The blot was then probed with the uniformly labelled probe as described in the Experimental section. The blots shown are representative of three blots of RNA isolated from three different groups of animals on separate occasions.

in rat fat-cells. Treatment of adrenalectomized animals *in vivo* with the potent glucocorticoid dexamethasone not only reversed the decline in G_{β} , but also increased this parameter by 50 % over the control values (Ros *et al.*, 1989). Glucocorticoids have been shown to regulate the steady-state levels of a variety of proteins, increasing their steady-state levels of mRNA (for review see Yamamoto, 1985).

We sought to investigate whether glucocorticoid regulation of G_{β} expression is exerted at the level of mRNA. In order to accomplish this, a synthetic probe was constructed and subcloned into bacteriophage M13 mp18 by the strategy of Linnenbach *et al.* (1986). This probe corresponds to nucleotides –10 to +60 of the human liver $G_{\beta 1}$ (Codina *et al.*, 1986). Based on computer analysis, the probe is predicted to recognize not only the mRNA of $G_{\beta 1}$, but also that of $G_{\beta 2}$, with which it shares 77 % identity at the nucleotide level. Fig. 1 (lane 1) displays an RNA blot (Northern) of total cellular RNA isolated from rat adipose tissue, validating this prediction. The probe recognizes two mRNA species, of 3.0 and 1.7 kb. The sizes of these mRNAs agree with the sizes for $G_{\beta 1}$ and $G_{\beta 2}$ mRNAs respectively, as deduced from RNA blots probed with nick-translated radio-labelled cDNA probes (Codina *et al.*, 1986; Fong *et al.*, 1987). The 1.7 kb mRNA could be detected only on relatively long autoradiographs, suggesting a lower abundance of this transcript in adipose tissue. This

Fig. 2. Effects of glucocorticoid status on the steady-state level of G_β mRNA in adipose tissue

Total cellular RNA (50 μg) isolated from adipose tissue from control (C), adrenalectomized (A), adrenalectomized dexamethasone-treated (AD) or control dexamethasone-treated (CD) rats, or single-stranded G_β M13 template DNA, was hybridized to the uniformly labelled probe and then digested with S1 endonuclease as described in the Experimental section. Panel (a) shows the standard curve obtained for S1-endonuclease-resistant hybrids by using increasing amounts of M13 template DNA. Panel (b) shows the effects of glucocorticoid status on the steady-state levels of G_β mRNAs in adipose tissue as measured by DNA-excess solution hybridization. The data, expressed as percentages of the control value, are means $\pm$ S.E.M. for three to five separate experiments performed with RNA isolated from separate groups of animals on separate occasions. Each determination was done in duplicate. The steady-state level of G_β mRNA in control animals was 5.8 ± 0.4 amol/ μg of total cellular RNA ($n = 5$). Values for CD and AD differed significantly ($P < 0.05$, Student's t test) from control (C) values. Likewise, the values for AD were significantly different from the values for A. In every experiment, a decrease in G_β mRNA after adrenalectomy was observed.

1.7 kb mRNA species has been shown to be of much lower abundance than the 3.0 kb species in several tissues examined, including liver and heart (Fong *et al.*, 1987).

RNA-blot analysis of total cellular RNA isolated from adipose tissue of control (C), adrenalectomized (A), adrenalectomized dexamethasone-treated (AD) and control dexamethasone-treated (CD) rats that was hybridized with the synthetic radiolabelled probe is displayed in Fig. 1 (lanes 2–5). The 3.0 kb species was prominent in RNA samples from all four sources, whereas the 1.7 kb species was not. RNA from the adrenalectomized rat (lane 3) displayed a slight decrease as compared with control animals (lane 2) in the 3.0 kb RNA species. This decrease in transcripts, though modest, was observed in several RNA blots. The levels of G_β mRNA in the dexamethasone-treated adrenalectomized rats (lane 4) were increased slightly with respect to controls (lane 2). Treating control animals with glucocorticoid alone did not appear to alter significantly the steady-state level of G_β subunit mRNA (lane 5).

From the RNA-blot analysis, the steady-state levels of mRNA for G-protein β -subunits appeared to be regulated by the glucocorticoid status of the animal. The difficulty in detecting the 1.7 kb G_β mRNA in this tissue even in long exposures of the blots, coupled with the limitations of sensitivity of Northern-blot analysis, necessitated the adoption of an alternative strategy for the quantification of G_β mRNA. DNA-excess solution-hybridization analysis, in addition to providing far greater sensitivity in the detection of low-abundance mRNAs than either RNA- or dot-blots, permits quantification of the amount of mRNA on a molar basis. This

technique has been successfully applied to the quantification of the steady-state mRNA of low-abundance G-protein-linked receptors (Hadcock & Malbon, 1988a,b).

By using DNA-excess solution hybridization and the M13 DNA template from which the probe was synthesized, a standard curve was generated. Uniformly labelled probe (2.4 fmol) was hybridized with increasing amounts of the template DNA. Hybridization was performed for 60 h at 68 $^\circ\text{C}$. The mixture was digested with the single-strand-specific endonuclease, S1, and the DNA–DNA hybrids that were resistant to digestion were precipitated with trichloroacetic acid, collected by vacuum filtration, and quantified by liquid-scintillation spectrometry. The standard curve obtained by plotting the amounts of S1-endonuclease-resistant hybrids versus the amounts of DNA template is shown in Fig. 2(a). The plot is linear over the range of template employed in the assay (0–2.4 fmol). The S1-endonuclease-resistance of the probe alone was less than 1% in these assays. From this standard curve, the steady-state levels of G_β -subunit mRNA could be quantified with precision.

The effects of adrenalectomy and dexamethasone treatment on the steady-state levels of G_β mRNA were analysed (Fig. 2b). The steady-state level of G_β mRNAs in control animals was found to be 5.8 ± 0.4 amol/ μg of total cellular RNA. As the synthetic DNA probe recognizes both the 3.0 kb and the 1.7 kb mRNA species (Fig. 1, lane 1), quantification represents that of the sum of both mRNAs. Adrenalectomy was associated with a 20% decline in the levels of mRNA for G_β . The levels of G_β mRNA were found to be decreased in each of four separate experiments. Dexamethasone treatment of the adrenalectomized animals overcame the effect of

Fig. 3. Effects of glucocorticoid status on the steady-state level of G_{sz} mRNA in adipose tissue

Total cellular RNA (50 μ g) isolated from adipose tissue from control (C), adrenalectomized (A), adrenalectomized dexamethasone-treated (AD), or control dexamethasone-treated (CD) rats, or single-stranded G_{sz} M13 template DNA were hybridized to the uniformly labelled probe and then digested with S1 endonuclease as described in the Experimental section. Panel (a) shows the standard curve obtained for S1-endonuclease-resistant hybrids by using increasing amounts of M13 template DNA. Panel (b) shows the effects of glucocorticoid status on steady-state levels of G_{sz} mRNA in adipose tissue, as measured by DNA-excess solution hybridization. The data, expressed as percentages of the control value, are means of two separate experiments performed with RNA isolated from separate groups of animals on separate occasions. The steady-state level of G_{sz} mRNA in control animals was 13.1 ± 0.7 amol/ μ g of total cellular RNA.

adrenalectomy, increasing G_{β} -mRNA levels to 130% of the control values. G_{β} -mRNA levels of glucocorticoid-treated adrenalectomized animals differed significantly ($P < 0.05$, $n = 4$) from those of adrenalectomized animals. Dexamethasone treatment alone increased G_{β} mRNA to approx. 150% of the control values [Fig. 2b, $P < 0.05$ for the difference ($n = 4$)]. The data obtained by DNA-excess solution hybridization confirm and extend the limited information gleaned from the RNA-blot analysis. These changes in the G_{β} mRNAs observed by using two independent techniques follow the same trend as those reported at the protein level (Ros *et al.*, 1989). Taken together, the data suggested that glucocorticoid regulation of G_{β} includes a component expressed at the level of mRNA.

The steady-state amount of G_{sz} mRNA was also determined by DNA-excess solution hybridization by using a probe with specificity for G_{sz} (P. J. Rapiejko, M. Ros, D. C. Watkins & C. C. Malbon, unpublished work). The amount of G_{sz} has been reported to be decreased by 30% at the protein level by adrenalectomy (Ros *et al.*, 1989). This decrease is reversed by treatment with dexamethasone (Ros *et al.*, 1989). Quantification of the mRNA encoding G_{sz} (Fig. 3) reveals the steady-state amount of these transcripts is not altered significantly by either adrenalectomy or the treatment of adrenalectomized animals with dexamethasone (Fig. 3b). In control cells, the G_{sz} mRNA is present at 13.1 ± 0.7 amol/ μ g of RNA. Whereas the changes in G_{β} associated with alterations in glucocorticoid status agree well with those observed at the level of steady-state expression of its mRNA, the same is not true for G_{sz} , suggesting a differential regulation of G-protein α - and β -subunits by glucocorticoids.

Alterations in expression of different G-protein subunits have been implicated in the altered hormonal

responsiveness that accompanies several endocrine imbalances. Hypothyroidism and adrenalectomy are good examples (Malbon *et al.*, 1985; Saggerson, 1986; Milligan *et al.*, 1987; Ros *et al.*, 1988, 1989; García-Sáinz *et al.*, 1989). Similarly, a decrease in the steady-state levels of G_{sz} by 38% and of G_{sz} mRNA by 30% appears to contribute to a heterologous desensitization in NG108-15 cells treated chronically with the xenobiotic, ethanol (Mochly-Rossen *et al.*, 1988). Glucocorticoids have 'permissive' effects on the action of catecholamines in many tissues, including rat fat-cells (Malbon *et al.*, 1988).

In the present paper, we demonstrate that glucocorticoids regulate the expression of the G_{β} subunits at the level of mRNA. G_{β} subunits are common to several G-proteins. Alteration of the steady-state levels of either G_{β} -subunits or G_{α} -subunits, or of the equilibria existing among these subunits, may provide the basis for the modulation of transmembrane signalling of several hormone-sensitive systems in a concerted fashion. Thus permissive hormones appear to offer diverse means of modulating information transduction across biological membranes, exerting influences on the mRNA levels of several elements in the system.

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