

# Paracrine glucocorticoid activity produced by mouse thymic epithelial cells

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**ABSTRACT** Previous data have suggested that glucocorticoids (GCs) are involved in the differentiation of thymocytes into mature T cells. In this report we demonstrate that the mouse thymic epithelial cells (TEC) express the cytochrome P450 hydroxylases Cyp11A1, Cyp21, and Cyp11B1. These enzymes, in combination with 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ HSD), convert cholesterol into corticosterone, the major GC in rodents. In addition, when TEC were cocultured with 'reporter cells' containing the glucocorticoid receptor (GR) and a GR-dependent reporter gene, a specific induction of reporter gene activity was observed. Induction of reporter gene activity was blocked when the TEC and reporter cells were incubated in the presence of the Cyp11B1 inhibitor metyrapone or the 3 $\beta$ HSD inhibitor trilostane, as well as by the GR antagonist RU486. Coculturing of TEC with thymocytes induced apoptosis in the latter, which was partially blocked by the enzyme inhibitors and RU486. We conclude that TEC secrete a GC hormone activity and suggest a paracrine role for this in thymocyte development.—Pazirandeh, A., Xue, Y., Rafter, I., Sjövall, J., Jondal, M., Okret, S. Paracrine glucocorticoid activity produced by mouse thymic epithelial cells. *FASEB J.* 13, 893–901 (1999)

*Key Words:* steroid biosynthesis • thymocyte development • cytochrome P450 • reporter cells • paracrine signaling

IN THE THYMUS, bone marrow-derived T cell precursors differentiate into mature T cells expressing T cell receptors (TCRs)<sup>2</sup> able to recognize self-MHC (major histocompatibility complex) (1). Double positive CD4/CD8 thymocytes with nonreactive or nonfunctional TCRs undergo apoptosis due to the lack of a TCR-mediated protective signal (default depletion due to neglect). This process represents the most extensive loss of thymocytes in the gland (2, 3). The thymus also eliminates self-reactive thymocytes, which carry TCRs that strongly interact with endogenous self-peptides presented by MHC (negative selection) (3). In contrast, thymocytes bearing TCRs

with low to moderate avidity for self-MHC/peptide complexes generate a protective signal that rescues the thymocytes from apoptosis (positive selection) (4, 5). The TCR-dependent positive selection process occurs in the cortical thymus through an intimate contact between developing thymocytes and TEC, whereas the negative selection process occurs in both the cortex and medulla, mostly on bone marrow-derived antigen-presenting cells (6–8). It is generally thought that the outcome of the thymocyte differentiation process (apoptosis or survival) is dependent on the strength of TCR-self-MHC/peptide reaction. However, it is not clear how these differences determine this outcome, although several investigators have suggested that glucocorticoids (GCs) may play a role in this selection process (9–14).

Thymocytes, particularly the immature double positive CD4/CD8 cells, are known to be highly sensitive to GCs, which readily induce apoptosis in these cells both *in vitro* and *in vivo* (15, 16). As the thymocytes are killed by apoptosis during the normal thymocyte development (3), we previously investigated whether GCs may be involved in the T cell differentiation process. We and others (9–11, 17, 18) have found evidence for this premise in studies of thymic apoptosis induced by anti-CD3 monoclonal antibody in normal mice (14) and by peptide in TCR

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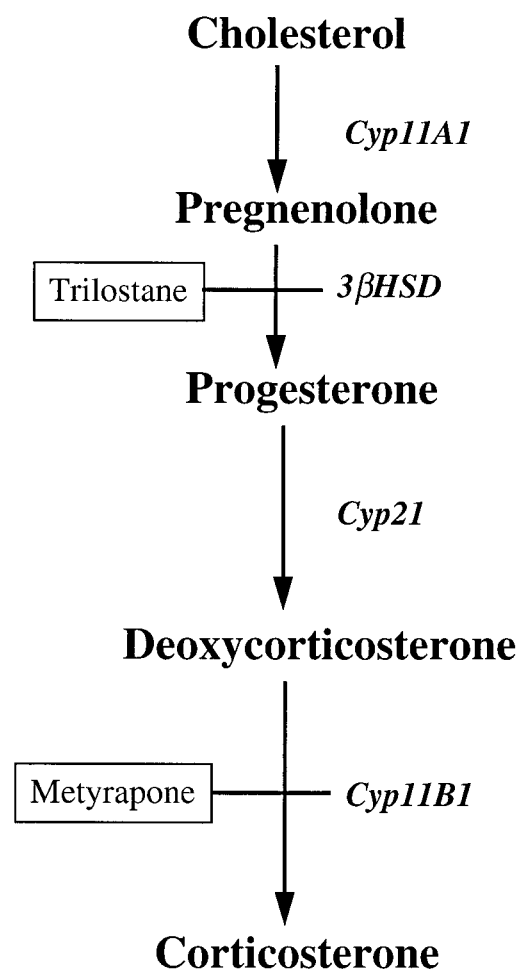
<sup>2</sup> Abbreviations: ACTH, adrenocorticotropic hormone; CRH, corticotropin-releasing hormone; GC(s), glucocorticoid(s); GR, glucocorticoid receptor; GREs, glucocorticoid response elements; HPA, hypothalamus-pituitary-adrenal; 3 $\beta$ HSD, 3 $\beta$ -hydroxysteroid dehydrogenase; metyrapone, 2-methyl-1,2-di-3-pyridyl-1-propanone; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TEC, thymic epithelial cells; TCR, T cell receptor; tk, thymidine kinase; trilostane, 4,5-epoxy-17-hydroxy-3-oxoandrostane-2-carbonitrile.

transgenic mice (12). Most important, we have found that the glucocorticoid receptor (GR) hormone antagonist RU486 inhibits thymic apoptosis in response to anti-CD3 and apoptosis induced by injection of a MHC-II binding peptide to peptide specific TCR transgenic mice (12). The protective effect by RU486 occurred both *in vivo* in adrenalectomized mice and in isolated thymic organ culture, excluding the possibility that RU486 antagonized GCs secreted from the adrenal gland. The protective effect by RU486 could be explained by the inactivation of GR activated by a GC-independent way or, alternatively, by protection from GC(s) produced in the thymus, as earlier suggested (17).

GC hormones are synthesized in the adrenal glands from the precursor cholesterol through sequential conversions by members of the cytochrome P450 superfamily of oxidases and 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ HSD) (19). Corticosterone is the main GC formed in rodents (Fig. 1). Its synthesis and release from the adrenals is under the control of the hypothalamus-pituitary-adrenal (HPA) axis, adrenocorticotropic hormone (ACTH) being the major mediator acting on the adrenals. When GCs are released into the bloodstream after ACTH stimulation, they distribute throughout the body, acting in an endocrine fashion on most tissues controlling e.g., metabolism, immune responses, and developmental processes. In addition to this endocrine signaling, locally produced steroids have been implicated in paracrine signaling. So-called 'neurosteroids' formed in the brain have been suggested to play a role both in cognitive functions and survival of cells in the nervous system (20–22). Furthermore, all enzymes required for the production of the major steroid hormones have been detected in the brain (23, 24). Locally produced GCs may also play a role in T cell development (10, 17). The thymus has endocrine features, e.g., it expresses some mediators of the HPA axis like corticotropin-releasing hormone (CRH) and ACTH (25–27).

GCs exert their effects via binding to the GR, a member of the nuclear receptor superfamily of intracellular receptors (28, 29). On binding, the hormone-receptor complex undergoes a process, called transformation, which includes dissociation of associated proteins and a conformational change. This permits the complex to interact with specific DNA sequences, termed glucocorticoid response elements (GREs), in target genes, which most often lie in the promoter region of regulated genes. Once bound to the GREs, the GR modulates (induces or represses) the activity of the target promoters. In some cases the GR modulates the activity of target genes not by directly interacting with GREs itself, but rather by binding to other transcription factors (28).

In the present work we have investigated the



**Figure 1.** Pathway of corticosterone biosynthesis. The figure depicts the steps in the steroidogenesis of corticosterone from the precursor cholesterol and the enzymes involved: cytochrome P450 side chain cleavage (Cyp11A1), 3-hydroxysteroid dehydrogenase/ $\Delta^5$ - $\Delta^4$  isomerase (3 $\beta$ HSD), cytochrome P450 21-hydroxylase (Cyp21), cytochrome P450 11 $\beta$ -hydroxylase (Cyp11B1). Action sites of the inhibitors trilostane and metyrapone (boxed) are shown.

capacity of cells present in the thymus to produce GC hormone activity. We have found expression of several steroidogenic cytochrome P450's in TEC. Furthermore, coculturing of TEC and 'reporter cells' containing GR and a GR-dependent reporter gene provided strong evidence that TEC produce a biologically active GC activity. This locally produced GC activity may contribute to the T cell differentiation at several different levels.

## MATERIALS AND METHODS

### Mice and tissue isolation

BALB/c mice were purchased from B & K Universal AB (Stockholm, Sweden), bred and housed under standard conditions in the animal facility at the Karolinska Institute, and used at an age of 4–5 wk. Tissues were removed and

prepared as described below. TEC were obtained from mice sublethally irradiated with 600 cGy 4 days before the removal of the thymus gland (17). Irradiated thymi were cut into small pieces by using extra-fine tip scissors, washed in phosphate-buffered saline (PBS), and put into Eppendorf tubes. Adrenal and muscle tissues were treated the same way. Isolated thymocyte single-cell suspensions were prepared by passage through a steel net. The thymocytes were washed in PBS and counted in trypan blue solution.

### Reagents and plasmids

RU486 was kindly supplied by Roussel-UCLAF, Center for Research (Romainville, France); rhodamine 123 was purchased from Sigma (St. Louis, Mo.). Metyrapone (2-methyl-1, 2-di-3-pyridyl-1-propanone) was bought from Aldrich Chem. Co. (Milwaukee, Wis.) and trilostane (4,5-epoxy-17-hydroxy-3-oxoandrostane-2-carbonitrile) was donated by Sanofi Winthrop (Newcastle, U.K.).

The reporter plasmid (GRE)<sub>2</sub>-tk-LUC, containing two GREs upstream of the heterologous thymidine kinase (tk) promoter and the luciferase reporter gene, has been described (30). The tk-LUC reporter gene lacks the two GREs. The GR expression vector used was SVGR1 (31).

### RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the tissues according to the method of Chomczynski and Sacchi (32) by homogenizing the tissue in 4 M guanidinium thiocyanate containing 25 mM sodium citrate pH 7.0, 0.5% (v/v) lauroyl sarcosine, and 0.1 M 2-mercaptoethanol, using a glass-to-glass homogenizer. Extreme care was taken to avoid cross-contamination between the samples. The quality of the RNA was examined by agarose gel electrophoresis after staining with ethidium bromide. RNA samples were stored as ethanol precipitates at -80°C.

First strand cDNA synthesis was carried out by incubating 2 µg of total RNA with 15 µM of the 3'-oligo primer (see below) in the presence of 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM DTT, 1 mM dNTPs, and 200 U of reverse transcriptase (Superscript RT, Life Technologies, Inc., Paisley, U.K.) for 1.5 h at 42°C in a total volume of 20 µl. The reaction was terminated by heating for 5 min at 95°C. Five microliters of this reaction were taken to a 20 µl PCR amplification reaction mixture containing 15 µM of the 5'-oligo primer (see below) in the presence of 10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.1% (v/v) Triton X-100, and 2 mM dNTPs. After denaturing at 95°C for 2 min and the addition of 2 U of *Taq* DNA polymerase (Promega, Madison, Wis.), PCR amplification was performed by 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min. A parallel reaction was carried out in the absence of reverse transcriptase to control for contamination with DNA. The primers used for reverse transcription and PCR amplification were as described previously by Stromstedt and Waterman (24) (all primers are given 5'→3'):

#### Cyp11A1

5'primer: CAA CAT CAC AGA GAT GCT GGC AGG

3'primer: CTC AGG CAT CAG GAT GAG GTT GAA

Length of product: 583bp

#### Cyp21:

5'primer: CTT CAC GAC TGT GTC CAG GAC TTG

3'primer: CAG CAG AGT GAA GGC CTG CAG CAG

Length of product: 775 bp

#### Cyp11B1:

5'primer: TCA CCA AAT GTA TCA AGA ATG TGT

3'primer: CCA TCT GCA CAT CCT CTT TCT CTT

Length of product: 666bp

### Southern blot analysis

Agarose gel electrophoresis of the PCR products and Southern blotting was performed as described (24). The oligonucleotides used as probes for detection of PCR products were as follows:

Cyp11A1: GGT GGA GTC TCA GTG TCT CCT TGA TGC TGG CTT TGA G

Cyp21: AAG GCC ACC AAG GGC ACC ACA GGC CGC AAA CGC AGC ACC T

Cyp11B1: CCT GCT GAA CAT CTG GGT TCC GAG CCA GCT CAA AAA G

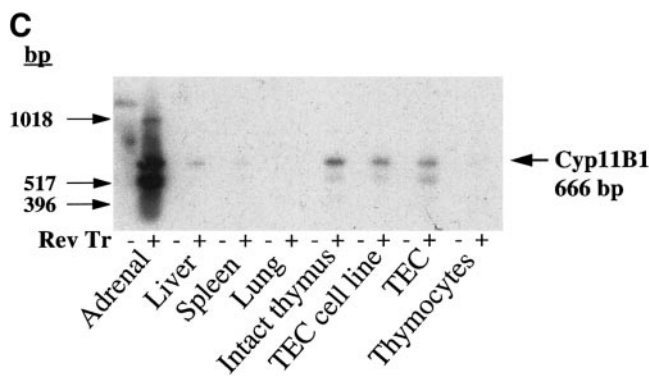
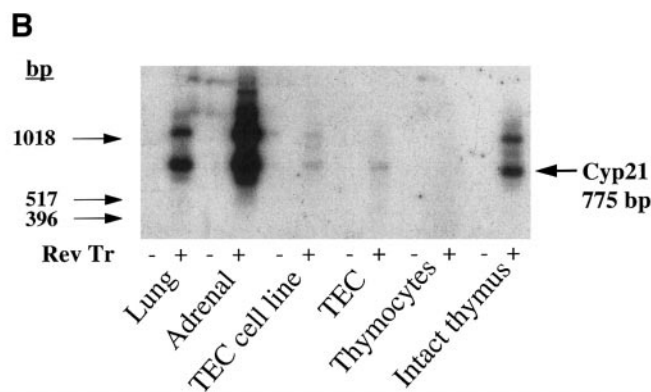
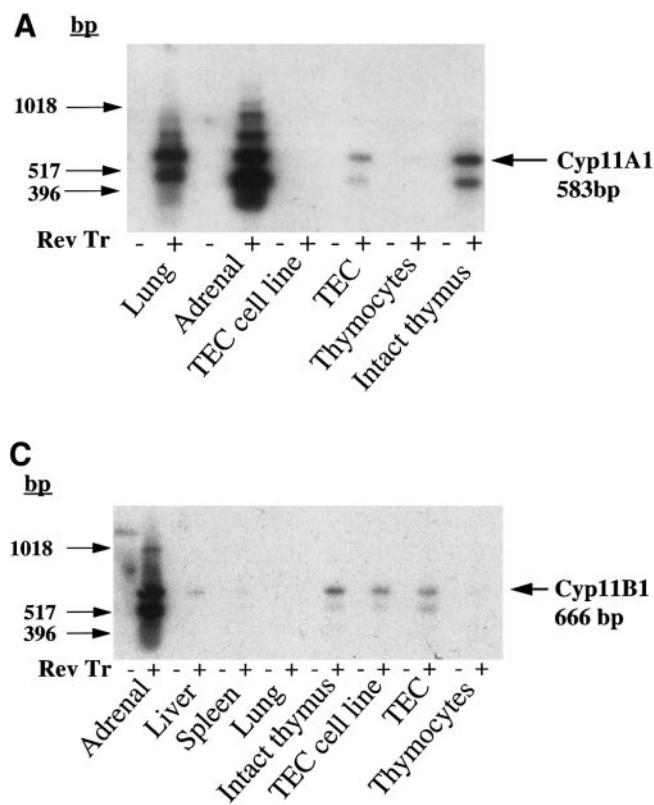
The oligonucleotides were end-labeled by T4 polynucleotide kinase using [ $\gamma$ -<sup>32</sup>P]ATP (3000 Ci/mmol, Amersham, Little Chalfont, U.K.).

### Western blot analysis

The tissues were homogenized in PBS, 20% (v/v) glycerol, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride by using a glass-to-glass homogenizer. The mitochondrial fraction was enriched according to Haaparanta et al. (33). Protein concentrations were determined by the Bradford assay (34) and the mitochondrial fraction was mixed with one volume of 2 × sodium dodecyl sulfate (SDS) buffer (100 mM Tris-HCl, pH 6.8, 0.2 M dithiothreitol, 4% SDS, 0.2% bromophenol blue, and 20% (v/v) glycerol); the indicated amount of protein was separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted to a C-Extra Hybond membrane (Amersham). Immunodetection was carried out by using the enhanced chemiluminescence detection kit from Amersham after incubation with the anti-Cyp11B1 antibody (35) at a dilution of 1:200, followed by a secondary horseradish peroxidase-labeled donkey anti-rabbit antibody (Amersham) according to the suggested protocols.

### Cell culture and transactivation experiments

COS-7 cells (obtained from American Tissue Culture Collection, Rockville, Md.) were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin at 37°C in 5% CO<sub>2</sub>. Cells were plated in 10 cm dishes at a density of 1 × 10<sup>6</sup> cells/plate 24 h before transfection. Cells were transiently transfected by using the calcium phosphate precipitation technique (36) with 5 µg of the reporter gene [(GRE)<sub>2</sub>-tk-LUC or tk-LUC] and 1 µg of the GR-expressing vector SVGR1. After overnight exposure of the cells to the DNA-calcium phosphate mixture, the cells were washed and allowed to recover for 5 h. Transfected COS-7 cells (reporter cells) were trypsinized, washed, and pooled from several plates. Two irradiated thymi (TEC) or two adrenals in an Eppendorf tube containing 0.5 ml culture medium were covered by 1 × 10<sup>6</sup> of pooled reporter cells in 0.5 ml of cell culture medium by a 2 min spin at 150 g. In some cases, an equivalent size of a piece of intact thymus, spleen, or skeletal muscle was used instead of the TEC. The addition of an equal number of transfected COS-7 cells from one pool avoided alterations in luciferase activity due to variation in transfection efficiency between the plates. If indicated, incubations were performed in the presence of 30 nM ACTH, 1 µM RU486, 50 nM trilostane, or 150 µg/ml metyrapone. In these cases, TEC were pretreated with the drug for 1 h before the addition of the COS-7 cells. After overnight incubation at 37°C in 5% CO<sub>2</sub>, cells were lysed and luciferase activity was



**Figure 2.** Expression of cytochrome P450 steroid hydroxylases in various tissues. Total RNA was prepared from various tissues and subjected to reverse transcription and PCR (RT-PCR) using primers for cytochrome P450 steroid hydroxylases involved in corticosterone biosynthesis (see Materials and Methods). Rev Tr - and + are PCR performed without or with prior reverse transcription. After PCR, products were separated on an agarose gel and subjected to Southern blotting using a  $^{32}\text{P}$ -labeled probe (independent from the primers used for the RT-PCR) for the different steroid hydroxylases. TEC and isolated thymocytes were obtained as described in Materials and Methods. The TEC cell line has been described previously (38). A) Cyp11A1, expected PCR product 583 bp. B) Cyp21, expected PCR product 775 bp. C) Cyp11B1, expected PCR product 666 bp.

determined. Calculation of luciferase activity was based on the total luciferase activity per tube, thus making it dependent only on added COS-7 reporter cells.

#### TEC-induced thymocyte apoptosis

TEC and thymocyte single-cell suspension were prepared as described above. TEC from one thymus or a piece of muscle of equivalent size were added to an Eppendorf tube and covered by  $1 \times 10^6$  thymocytes in 1 ml of culture medium. When indicated, incubations were performed in the presence of 1  $\mu\text{M}$  RU486, 50 nM trilostane, or 150  $\mu\text{g}/\text{ml}$  metyrapone. In these cases, the drug was added to the TEC 1 h before the addition of the thymocytes. After 16 h of incubation at 37°C, thymocytes were stained with 2.5 mM rhodamine 123 for 60 min (37). Cells were then washed with cold PBS and 10,000 gated cells were directly analyzed using a FACScan (Becton Dickinson, Rutherford, N.J.).

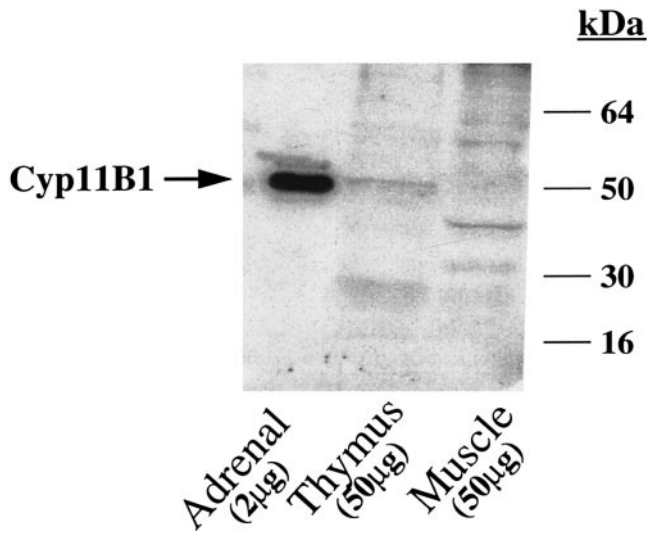
## RESULTS

### Enzymes involved in corticosterone biosynthesis are expressed in the mouse thymus

The enzymes involved in corticosterone synthesis from cholesterol are indicated in Fig. 1. The first and rate-limiting step in corticosterone synthesis is the conversion of cholesterol to pregnenolone (19). This reaction is catalyzed by the cytochrome P450 side chain cleavage enzyme Cyp11A1 (19). Using RT-PCR, we detected Cyp11A1 mRNA in total RNA extracts prepared from intact thymi (Fig. 2A). Although not quantitative, the Cyp11A1 signal from

thymus was less intense than the Cyp11A1 signal from adrenal, indicating that the Cyp11A1 expression level is lower in the thymus. Cyp11A1 expression was also found in the lung (Fig. 2A). When the thymus was split into the thymocyte and TEC compartments, it was clear that Cyp11A1 expression was confined to the TEC compartment (Fig. 2A). In contrast, no Cyp11A1 expression was found in a TEC cell line (38). Also, the cytochrome P450 21-hydroxylase (Cyp21) and the P450 11 $\beta$ -hydroxylase (Cyp11B1) mRNAs were detected in the mouse thymus (Fig. 2B and C, respectively). In both cases, expression was restricted to the TEC compartment, with no evidence for expression in the thymocytes. In contrast to Cyp11A1, both Cyp21 and Cyp11B1 were detected in the TEC cell line. Cyp21 was also detected in the lung, whereas Cyp11B1 was detected in the liver but not in the lung or in the spleen.

To investigate whether the mRNA expression also resulted in protein expression, a Western blot analysis of Cyp11B1 expression was performed. As can be seen from Fig. 3, a weak Cyp11B1 expression was detected in the thymus. In contrast, no Cyp11B1 was detected in the muscle. The Cyp11B1 signal in the thymus was at least 50-fold less intense than the signal from the adrenal. Taking into account that the amount of thymic mitochondrial protein applied was 25-fold more, the expression of Cyp11B1 in the thymus is at least 1000-fold lower than the expression in the adrenal.

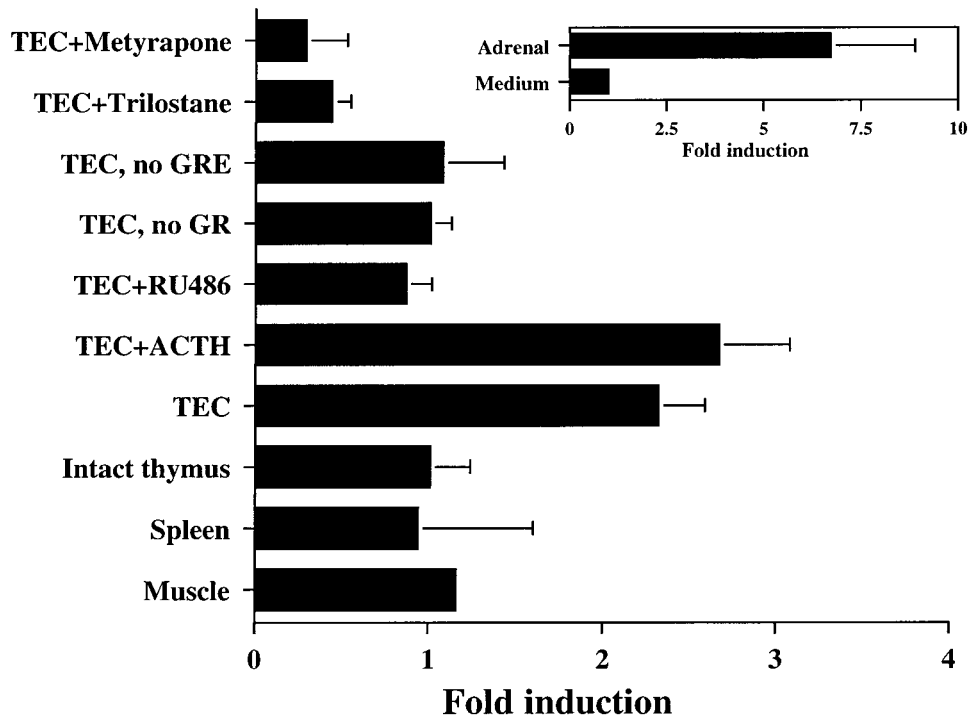


**Figure 3.** Western blot analysis of Cyp11B1 expression in the thymus. The indicated amount of mitochondrial protein from adrenal, skeletal muscle and thymus was subjected to SDS-PAGE analysis and Western blotting using an anti-Cyp11B1 antibody as described in Materials and Methods.

### TEC secrete a factor that activates a GC-regulated reporter gene in cocultured cells

To test whether TEC not only express the required enzymes for corticosterone production but indeed produce a biologically active GC activity, TEC were cocultured with COS-7 cells that had been transfected with a GR expression vector and GRE-luciferase reporter gene. This coculturing resulted in a  $2.3 \pm 0.3$ -fold induction ( $n=9$ ) of luciferase activity (Fig. 4). This induction could be compared with the sevenfold increase observed when the reporter cells were incubated with adrenals (Fig. 4, insert). The luciferase induction induced by the coculturing with TEC was significant ( $P<0.001$ ). No induction of luciferase activity was observed when the reporter cells were incubated with muscle, spleen, or intact thymus (Fig. 4). We observed a slight but not significant enhancement of luciferase activity when ACTH was added to the TEC during the coculturing.

The luciferase induction was totally blocked when the GC antagonist RU486 was added to the incubation (Fig. 4). Furthermore, the induction of the reporter gene activity required the presence of the GR in the reporter cells, since no induction was observed in experiments in which the receptor was

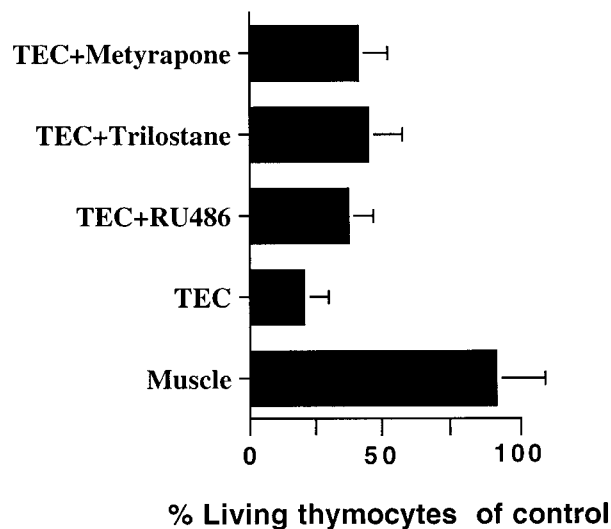


**Figure 4.** Reporter cells cocultured with TEC identify a secreted factor able to induce a GC-regulated reporter gene. COS-7 cells transfected with an expression vector for the GR and the  $(GRE)_2$ -tk-luciferase reporter gene 'reporter cells' were cocultured with TEC or various other tissues overnight and reporter gene activity was analyzed. In some cases, hormones (30 nM ACTH, 1  $\mu$ M RU486), inhibitors of  $3\beta$ HSD (trilostane, 50 nM), or Cyp11B1 (metyrapone, 150  $\mu$ g/ml) were added to the TEC 1 h before as well as during the incubation with the reporter cells. In some cases, the reporter cells lacked the GR expression vector (no GR) or the GREs in the reporter gene (no GRE) needed to confirm a GR-dependent transcriptional activation of the reporter gene. Fold induction is expressed relative to the activity of the reporter gene when reporter cells were incubated with medium alone. Values are mean  $\pm$  SD from 2–5 experiments. For adrenal,  $n=10$ ; muscle,  $n=2$ ; spleen,  $n=6$ ; intact thymus,  $n=7$ ; TEC,  $n=9$ ; TEC+ACTH,  $n=6$ ; TEC+RU486,  $n=4$ ; TEC no GR, TEC no GRE, TEC+Trilostane, TEC+Metyrapone, all  $n=3$ .

omitted from the COS-7 cells. In addition, the presence of the GREs in the reporter gene was also required, because no induction was seen when the reporter gene lacked the GREs. Finally, inclusion in the coculture of the  $\beta$ 3HSD inhibitor trilostane or the Cyp11B1 inhibitor metyrapone prevented the induction of the reporter gene (Fig. 4). The reason for a low basal luciferase activity generated when COS-7 cells were incubated with trilostane or metyrapone is unclear, but is not due to a toxic effect on the COS-7 reporter cells. In addition, activation of the reporter gene in the COS-7 cells by dexamethasone is unaffected by the concentrations of trilostane or metyrapone used (data not shown). In summary, the results demonstrated that the induction is a true GR-mediated response due to the production of a GC hormone activity, most likely a GC, in the TEC.

### GC activity produced in the TEC contributes to thymocyte apoptosis

We have previously demonstrated that the GC antagonist RU486 can partially prevent anti-CD3 or peptide-induced apoptosis in thymic organ cultures of TCR transgenic mice (12). This may suggest that an endogenously produced GC hormone activity cooperates with the anti-CD3 and peptide effect. Furthermore, fetal thymic organ cultures (39) or experiments in which GC sensitivity in the thymocytes has been changed by repressing endogenous GR expression or overexpressing the GR have demonstrated that GCs play a role in T cell development (18, 40; A. Pazirandeh et al., unpublished results). To test whether the GC activity locally produced in the thymus can influence thymocyte apoptosis, TEC were cocultured with freshly isolated thymocytes. As can be seen in Fig. 5, 23% of the thymocytes were alive after 16 h of coculturing with TEC as compared with the number of living thymocytes incubated for the same time in medium alone. In contrast, ~90% of the thymocytes were alive when cocultured with a piece of skeletal muscle unable to produce the GC activity (c.f. Fig. 4 above). The thymocyte population mainly affected by the coculturing with the TEC was the CD4<sup>+</sup>/CD8<sup>+</sup> double positive cells, as analyzed by FACS (data not shown). The inclusion of the GC antagonist RU486 or the steroid synthesis inhibitors, trilostane and metyrapone, all partially protected ( $P < 0.05$ ) against TEC-induced apoptosis of the thymocytes (Fig. 5). The percentage of surviving thymocytes doubled from 23% to ~41–48% after treatment with any of these agents. In the absence of TEC, RU486, trilostane, or metyrapone by themselves in the concentrations used did not influence thymocyte survival (data not shown). This demonstrates that the GC activity produced locally in the thymus affect thymocyte survival.



**Figure 5.** Thymocytes cocultured with TEC undergo apoptosis and are partially protected by a GC antagonist (RU486) or inhibitors of steroidogenesis.  $1 \times 10^6$  freshly isolated thymocytes were cocultured with a piece of skeletal muscle or TEC for 16 h. The thymocytes were isolated, stained with rhodamine 123, and analyzed by FACScan for the percentage of living and apoptotic cells. When indicated, hormone (1  $\mu$ M RU486), inhibitors of  $\beta$ 3HSD (trilostane, 50 nM), or Cyp11B1 (metyrapone, 150  $\mu$ g/ml) were added to the TEC 1 h before as well as during incubation with the thymocytes. The number of living thymocytes was expressed relative to the number of living thymocytes cultured for 16 h in medium alone, the latter serving as the control. Values are mean  $\pm$  SD ( $n=3$ ).

### DISCUSSION

We have found that TEC express steroidogenic cytochrome P450 hydroxylases and can activate in a paracrine fashion a GR-dependent reporter gene in cocultured cells or induce apoptosis of coincubated thymocytes. The TEC-produced factor can be defined as a GC based on its ability to activate a GC controlled reporter gene. This is based on the requirement for the presence of the GR in the reporter cell as well as a GRE in the reporter gene (Fig. 4). Furthermore, the activity is blocked by the GR antagonist RU486, which competes with GC agonists for binding to the same ligand binding pocket in the GR. Although we have not so far established the exact chemical nature of the effector molecule, several experimental results suggest that the TEC produce and secrete GC hormone(s) that can influence the surrounding thymocytes. The RT-PCR demonstrated the expression, albeit in low levels, of mRNAs for the required hydroxylases involved in corticosterone synthesis. The presence of  $\beta$ 3HSD activity, the additional enzyme activity required for corticosterone biosynthesis (Fig. 1), has been demonstrated in separate studies. Thus, TEC, but not thymocytes, were found to generate 20 $\alpha$ -hydroxy-4-pregnen-3-one from added pregnenolone, a conversion requiring participation of a  $\beta$ 3HSD (J. Zhang, Y.

Xue, Yang, S. Okret, M. Jondal, and J. Sjövall, unpublished results). Activation of the target gene in the coculture assay is blocked by inhibitors of enzymes involved in steroidogenesis. In addition, incubation of TEC with pregnenolone generated supernatants from which material was extracted and separated by high-performance liquid chromatography into an expected corticosterone fraction. This fraction was found to induce apoptosis of thymocytes, inhibitable by the GC antagonist RU486 (data not shown). Similar to Vaccio et al. (17), we could detect some immunoreactivity using a radioimmunoassay for deoxycorticosterone. However, the specificity of the assay was interfered by the addition of the high concentration of the precursor. Furthermore, the administration *in vitro* of a high concentration of a precursor does not prove the production of GCs *in vivo* when physiological levels of precursors are present. Instead, we are trying to conclusively identify the active compound(s) in the absence of precursor administration at the femtomol level by using mass spectrometric methods.

Several experimental conditions used in the RT-PCR guaranteed that the expression of the P450 steroid hydroxylases in the TEC population is a true finding and not due to contamination of genomic DNA. First, no expression was detected in the absence of reverse transcriptase; second, primers used in the PCR were localized in different exons, thus utilizing the expected cDNA size of the produced band as an internal control. Finally, a probe independent of the PCR primers was used to detect the expected product by Southern blotting. In addition, P450 11 $\beta$ -hydroxylase (Cyp11B1), the final enzyme involved in the biosynthesis of corticosterone, is detected by Western blotting. The reason for the additional bands seen in the Southern blot after the RT-PCR, particularly in the highly expressing tissues, is most likely a conversion of PCR products to random-length, higher molecular weight fragments when a high number of PCR cycles was used to detect the products in the low-expressing tissues (41). It is important to note that the expression of the cytochrome P450 steroid hydroxylases in TEC was not a consequence of the irradiation of the mice, since expression was also detected in intact thymi from nonirradiated mice. Furthermore, despite the presence of enzymes involved in the classical biosynthetic pathway of glucocorticoids, one also has to consider a possible nonclassical biosynthesis pathway, as has been described for the formation of some steroids in brain tumor cells (42).

The coculturing experiments between TEC and a reporter cell line containing a GR-dependent reporter gene demonstrated that the TEC secrete enough of GC hormone activity to influence a neighboring cell. In contrast, other tissues like the spleen

or muscle were unable to induce the reporter gene. Thus, the induction of the reporter gene was not a consequence of serum in the medium or blood contamination, since all the tissues were in the same medium and some of the tissues (e.g., muscle and spleen) contained more blood than the TEC. When incubating a piece of an intact thymus with the reporter cell line, no induction of the reporter gene was seen. This may reflect that the TEC-secreted GC activity only acts in a paracrine fashion and only cells in its close proximity will be affected. This reflects a difficulty in general for the analysis of paracrine systems where local concentrations can be high but overall concentrations are very low. Thus, determination of the concentration of the activity in medium or an extract will not reveal the local concentration. As paracrine interactions are likely to be important for the function of many tissues, a cocultured reporter system, as used by us, is advantageous in revealing a physiologically significant local hormone production. Such cocultured 'reporter systems' have become an important detection tool in studies of the formation of low concentrations of bioactive molecules (43). That the GC activity produced locally in the thymus indeed may have an important role in T cell development is suggested by the demonstration that it contributes to the apoptotic activity on thymocytes (Fig. 5). Of interest is that incubation of a TEC cell line, in which we detected some of the P450 steroid hydroxylases (Fig. 2), with RU486 protects cocultured immature CD4<sup>+</sup>8<sup>+</sup>3<sup>+</sup> thymocytes from apoptosis (38). The reason for the incapability of RU486, trilostane, or metyrapone to fully protect thymocyte apoptosis in the TEC-thymocyte coculture assay is not clear, but could be due to an inability of the metabolic inhibitors to fully block GC biosynthesis (a nonclassical biosynthesis pathway, as discussed above), a partial agonistic activity of RU486 (44), or other GC-independent mechanisms in the TEC-thymocyte interaction may contribute to the degree of thymocyte apoptosis (45).

Earlier reports support the idea that GCs are important for T cell development in the thymus (9–14). A direct role for GCs in thymocyte development has been formulated as the 'mutual antagonism' model, which states that GCs oppose apoptotic signals generated by medium avidity TCR stimulation and lead to positive selection (9, 10). Negative selection is explained as the inability of GCs to counteract a strong TCR signal generated through high-avidity recognition of self-MHC/peptide complexes. Evidence for this hypothesis originates from experiments done in thymic tissue cultures and in GR antisense transgenic mice (9, 10, 40). In GR antisense transgenic mice, which have a reduced number of GRs in the thymocytes and thus an impaired GC sensitivity, it was found that the size of

the thymus was reduced, mainly as a consequence of reduced number of CD4/CD8 double positive thymocytes (40). However, experiments from another group as well as ourselves using the same antisense GR transgene, under the control of a different or the same promoter, found the opposite effect (18; A. Pazirandeh et al., unpublished results).

Our data support a model in which GCs, at a given concentration, primarily have an apoptotic effect on immature thymocytes. Positive selection would then be explained by protective TCR signals, possibly associated with signals generated in the MAP kinase pathway, which might down-regulate GR responsiveness (46, 47). Alternative signals, generated by high-avidity TCR recognition of self-MHC/peptide complexes, might have the opposite effect, e.g., to increase GC responsiveness (13). To support this, we have found that the GC antagonist RU486 inhibits thymic apoptosis induced by the OVA323–339 peptide in DO11.10 TCR transgenic mice (12). In the same DO11.10 mouse strain, we also find a strong positive selection effect of I-A<sup>d</sup>, in combination with undefined endogenous self-peptides, which is related to a relative GC resistance in the transgenic thymocytes and to a reduced GR responsiveness *in vitro* (unpublished results). In addition, when we increased GC sensitivity in normal mouse thymus by overexpressing (twofold) the GR under the control of the p56<sup>lck</sup> proximal promoter, we found a reduced thymocyte number and a diminished number of mature T cells in the periphery (unpublished results). The function of GC activity in thymus could also be considered in terms of the well-known regulatory effect of the HPA axis in the peripheral immune system (11, 48). In thymic tissue, both CRH and ACTH are expressed (25, 26, 49) and thus might be part of a local HPA axis potentially important for regulating the size of the organ, as well as applying a negative pressure during T cell differentiation. The strength of this negative pressure might be important in shaping the T cell repertoire and for the development of autoimmune diseases, as has been demonstrated for the HPA axis in several animal autoimmune disease models.

In summary, although the exact function of thymic GC exposure is equivocal, data clearly suggest that TEC do produce a biologically relevant GC activity that can affect surrounding thymocytes. FJ

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