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Expression of hsp90β Messenger Ribonucleic Acid in Patients with Familial Glucocorticoid Resistance—Correlation to Receptor Status

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We have previously shown an increased specific DNA-binding of liganded unactivated glucocorticoid receptor (GR) to the LTR-region of MMTV DNA in a patient with primary cortisol resistance and receptor thermolability indicating a defective interaction of GR with hsp90. In some patients, however, no apparent receptor abnormality was found in spite of a characteristic phenotype. mRNA expression levels of hsp90β were analysed in cultured fibroblasts from patients with known receptor defects, such as thermolability, decreased ligand binding affinity and low receptor expression levels, and from patients with a cortisol resistant phenotype but no detected receptor alteration. Fibroblasts from patients with GR defects expressed higher hsp90β mRNA levels as compared to patients with no receptor defects or to healthy controls. These data indicate that GR defects are associated with increased hsp90β mRNA levels.

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INTRODUCTION

Physiological stress, such as heat-shock, preferentially enhances the synthesis of a limited number of intracellular proteins (heat shock proteins or hsps) [1]. This response has been observed in all cells so far tested and some of the hsps are highly conserved across species [2]. The most strongly heat-inducible and conserved hsps found in eukaryotic cells are proteins with molecular weights of about 90 kDa (hsp90) and 70 kDa (hsp70). hsp90 and hsp70 are abundant intracellular proteins and it is likely that they have important roles in normal cellular function in addition to the role they seem to play in cellular adaptation to stress. Recently, hsps have been suggested to be of significance for the immune response, such as in connection with intracellular antigen processing [3] and the presentation of cell membrane-anchored antigens to the immune system [4]. Furthermore, elevated levels of hsp90 have been reported in a proportion of systemic lupus erythematosus (SLE) patients with active disease [5].

In addition, evidence for a role of hsp90 in the action of steroid hormone receptors has continued to accumulate. Current models of transcriptional activation by steroid hormone receptors reflect general acceptance of the importance of hsp90 and incorporate it in the unactivated receptor structure [6]. The unliganded GR is now believed to be a heteromer composed of a single steroid- and DNA-binding subunit and two hsp90 subunits [7]. According to this model hsp90 has a dual function in the hormone dependent conversion of a transcriptional regulatory protein, such as the glucocorticoid receptor (GR), from an inactive to an active state: (i) hsp90 seems to stabilize GR in a conformation, which is necessary for high affinity ligand binding; and (ii) hsp90 is also important in maintaining GR in a non-activated form, i.e. non-DNA-binding form [8].

Theoretically, any change in hsp90 structure and function or in receptor subunits interacting with hsp90 might affect the specific DNA-binding of GR and

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influence the biological action of the receptor. Studies of patients with primary cortisol resistance or spontaneous hypercortisolism without Cushing's disease have revealed a number of GR defects, such as thermolability, altered ligand affinity and decreased number of receptors, which in a few cases have been explained in terms of specific mutations within the steroid binding domain of the receptor [9–11]. These mutations are likely to alter the interaction of the receptor protein with hsp90. However, in some of the patients no apparent receptor abnormality was found although the criteria of the disorder were fulfilled [12]. We have previously reported specific DNA-binding of liganded GR from a patient with GR thermolability and primary cortisol resistance already at 4°C where GR does not normally bind specifically to DNA, indicating a defective interaction between GR and hsp90 [13]. Unpublished results (Brönnegård *et al.*) indicated a mutation in the steroid binding domain of GR from this patient. The present study is aimed at investigating a possible correlation between basal expression of hsp90 mRNA and the presence of GR defects in patients with primary cortisol resistance.

MATERIALS AND METHODS

Patients included all had hypercortisolism and pathological dexamethasone suppression/ACTH stimulation tests, and had no clinical signs of Cushing's disease. Clinical characteristics and receptor defects of the study group have previously been reported [12]. All together, 16 patients were included. Seven of these patients had different glucocorticoid receptor defects, such as thermolability, decreased binding sites per cell or altered K_D-values and we have previously shown that the cultured fibroblasts retain the glucocorticoid resistance state of the patients [12, 13]. In the remaining nine patients, however, no receptor defect could be found. Fibroblasts from six healthy controls with normal receptor characteristics were also included.

RNA preparation and probe synthesis

Fibroblasts were established from explants of skin and total RNA prepared by the guanidine thiocyanate water-saturated phenol/chloroform extraction method [14]. Cells were washed with PBS, dissolved in guanidinium thiocyanate solution and then homogenized. RNA from these patients was hybridized with a 2.6 kb full-length cDNA probe of the human hsp90 gene [15]. Probes for β-actin [16] and GAPDH were used as internal controls. The cRNA probe for GAPDH corresponded to the first 50 bases in exon 4 [17]. The probes were labeled by ^{32}P-dCTP (3000 Ci/mmol) to a specific activity of $2–6 \times 10^7$ cpm/μg according to the nick-translation technique [18].

Northern blot and slot blots

The expression of hsp90 was analysed by Northern blot and slot blot hybridization of total RNA [18]. The same slot blot was washed free of hsp90 probe and successively rehybridized to GAPDH or β-actin probes, respectively. The slot blot experiments were performed twice. The signal intensity obtained with the GAPDH and β-actin probes in each slot blot was correlated to the hsp90 signal, providing a control for RNA content among samples. The integrity, quality and amount of each sample were measured by a preliminary electrophoresic analysis in 0.9% agarose gel after ethidium bromide staining (data not shown). The total amount and quality of RNA were also determined spectrophotometrically at 260 and 280 nm, respectively.

For Northern blots, denatured total RNA samples (25 μg) were fractioned on a 0.9% formaldehyde agarose gel and transferred to a nitrocellulose filter. The filter was prehybridized and hybridized to the ^{32}P-labeled probes under stringent conditions as previously described [18]. Filters were dried and exposed at −70°C to XAR 5 Kodak films. For slot blots, nitrocellulose was prewashed with millipore redistilled water and applied to a slot blot apparatus (Schleicher and Schwell). The slot for each sample contained 10 μg of total RNA. The slot blots were prehybridized, hybridized and washed as described for Northern blots. Signal intensities of slot blots were assessed by densitometric scanning and results presented as arbitrary units (AU) of the scannings. Each result presented has been adjusted to the expression of GAPDH in order to make results obtained comparable between the samples investigated.

RESULTS

All patients and the six controls expressed hsp90β mRNA. Probe specificity was demonstrated by Northern blot analysis where one control (No. 1) and patients No. 10 and 18 were shown to express a hsp90β mRNA transcript of corresponding size (2.6 kb) (Fig. 1). Figure 1 also shows equal expression of GAPDH mRNA (GAP) in these three individuals. In this study, we found that β-actin was differently expressed in all cases and therefore not valid as an internal control (data not shown). The use of GAPDH as an internal control, however, indicated that the results from analysis of hsp90β mRNA expression levels were comparable. GAPDH gene expression was not influenced by disease or GR-defects as indicated by the same relative GAPDH mRNA levels to total RNA. Therefore, results from slot blot analysis of hsp90β were normalized to the expression of GAPDH mRNA and not to β-actin, and presented as arbitrary units.

The basal expression of hsp90β mRNA was increased in 14 out of 16 patients investigated as com-

pared to the controls [Fig. 2(a)]. In Fig. 2(b) the corresponding control hybridization signals for GAPDH (GAP) mRNA are shown. We have previously shown that manifestations of primary cortisol resistance vary from six of seven manifestations in one patient down to two of seven in another [12]. As indicated in Fig. 2(c), there is a tendency of higher hsp90β mRNA expression levels in patients with two or more manifestations. We have previously shown that patients 3, 4, 6, 9, 10, and 14–16 all have GR-defects [12], in contrast to patients 5, 7, 8, 11–13 and 17–18 (patients 11 and 18 did not show a significant increase in hsp90β mRNA expression). The latter patients, however, all have hypercortisolism and pathological dexamethasone suppression/ACTH-stimulation tests but no Cushingoid phenotype. Patient No. 4 is the son of patient No. 3 and has been shown to have a thermolabile GR [19]. In Fig. 2(c) the number of GR-related abnormalities in relation to the expression of hsp90 mRNA is shown, indicating a correlation between receptor status and hsp90 mRNA levels. When the six controls and the eight patients where no GR-defect was found were compared to the patients with GR-defects a significant increase in hsp90 mRNA was observed ($P > 0.01$; Mann–Whitney U-test). When the eight patients with no GR-defects were compared to the six controls, however, no significant increase in hsp90 mRNA was found.

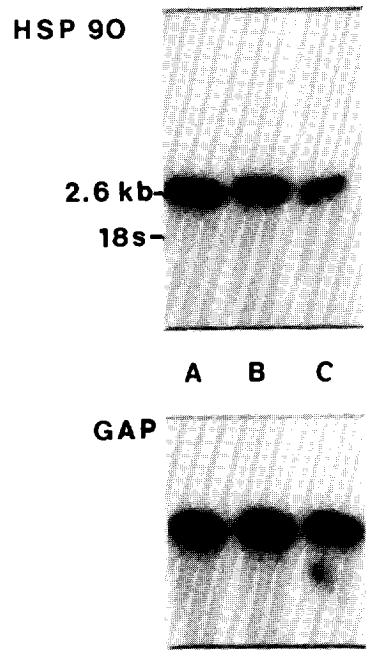

Fig. 1. Analysis of hsp90 and GAPDH mRNA transcripts in fibroblasts from patient No. 10 (A) and No. 18 (B) and a control (C) by Northern blot hybridization. Position of RNA market is indicated.

DISCUSSION

Substantial evidence exists for a role of hsps in the signal transduction pathway for steroid hormones in health as well as disease [20]. It has been shown that reduced levels of hsp90 compromise steroid receptor action *in vivo* [21] and specific heat shock proteins have been implied as a link between the immune response to infection and autoimmunity [3, 4]. At the cellular level hsp90 interacts not only with steroid hormone receptors but also with tyrosine and serine-threonine kinases [22] and other hsps but its biological functions are not fully understood.

Steroid hormone receptors contain a conserved sequence of amino acids within the steroid binding domain which is the site of interaction of the receptor with hsp90 [23]. The exact sites of hsp90 binding have not yet been established but a number of reports confirm that binding of hsp90 helps to stabilize the GR against proteolysis [6]. Furthermore, deletion experiments have shown that the steroid binding domain contains the features that determine both repression of DNA binding activity and steroid-mediated derepression of DNA binding activity [23]. The mechanisms by which the steroid binding domain represses function and by which the binding of hormone leads to depression of function might thus involve the association of hsp90 with the receptor.

We have previously shown that liganded GR from a patient with familial glucocorticoid resistance (FGR), in contrast to liganded control GR, bound specifically to MMTV DNA in the absence of heat-treatment, indicating an altered interaction between hsp90 and FGR–GR [13]. Results presented in this study also show that hsp90 mRNA levels are significantly increased in FGR-patients and that there is a correlation between receptor status and hsp90 mRNA expression levels. It is therefore possible that an alteration in GR causes a "stress situation", whereby increased synthesis of hsp90 prevents an excess degradation of the defective receptor protein thereby helping to maintain the homeostasis of the cell. On the other hand, the mutant receptor described by Housley *et al.* [23] undergoes extensive intracellular cleavage and, in spite of being bound to hsp90, the complex appears to be much less stable than that of the wild-type receptor. A general function suggested for hsp90 is to stabilize proteins against degradation in the cell and to exert some type of chaperone function related to protein folding [6].

Results presented in this study represent the first report on differential expression of hsp90 mRNA in fibroblasts from patients with familial cortisol resistance. Further studies are needed to evaluate the role of hsp90 in steroid hormone resistance syndromes and to determine the exact sites of interaction of hGR with hsp90. Structural alterations within these regions of the GR gene in patients with FGR might influence both hsp90 mediated repression of DNA binding activity and steroid-mediated derepression of DNA binding activity.

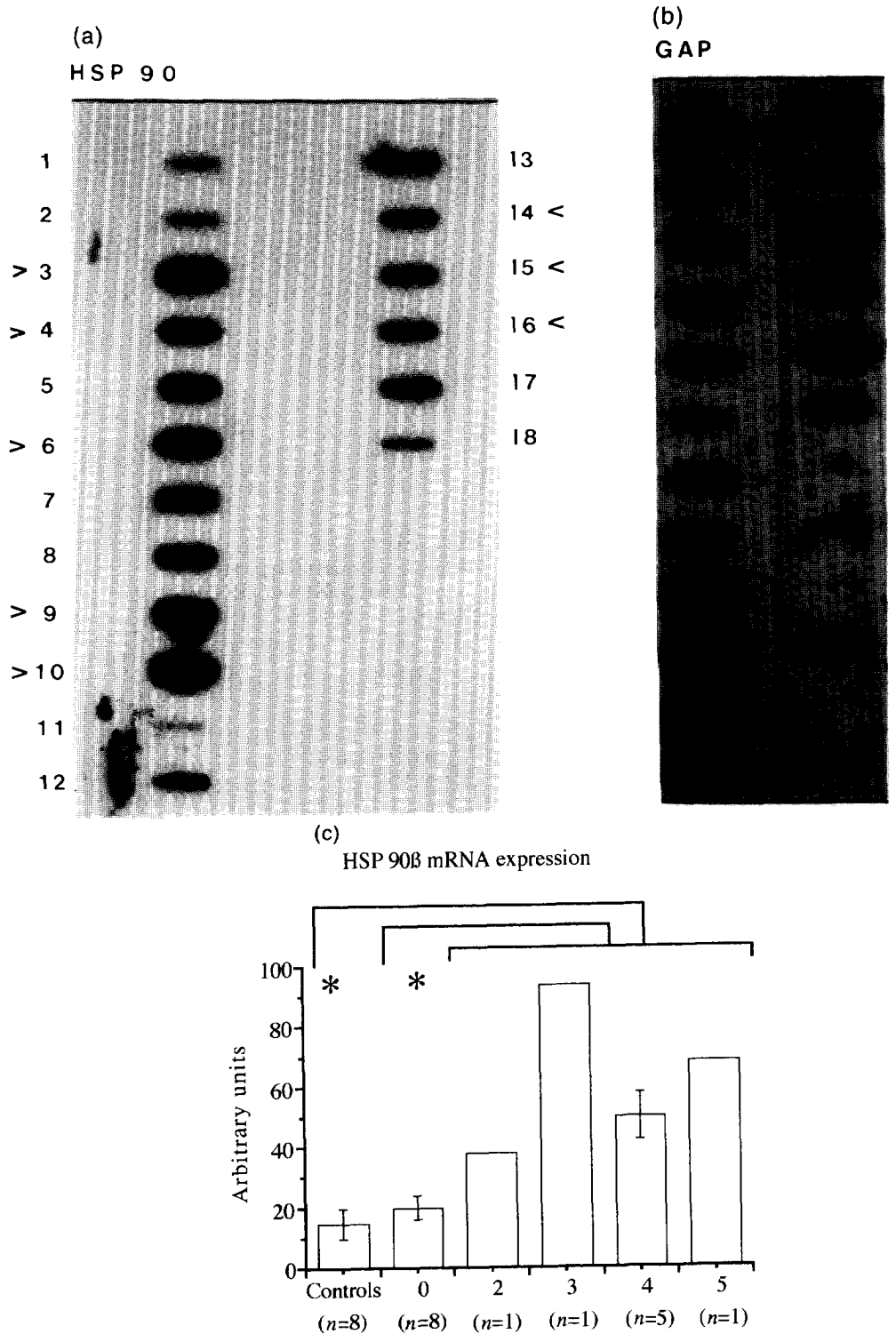

Fig. 2. Slot blot analysis of hsp90β and GAPDH mRNA expression in fibroblasts. (a) hsp90β, hybridization with RNA samples of 10 μg from two controls (1 and 2) and 16 patients (3–18) with primary glucocorticoid resistance. Patients with GR-defects are indicated (>). (b) Corresponding hybridization using the GAPDH cRNA probe. (c) Densitometric scanning data from slot blot analysis as compared to the number of GR-defects found (0–5). Results are expressed as arbitrary units after normalization to the expression of GAPDH mRNA. Significant differences ($P < 0.01$) between control and patients with GR-defects and between patients with hypercortisolism but no found GR-defect and patients with GR-defects are indicated (*).

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