Interleukin-4 upregulates the heat shock protein Hsp90α and enhances transcription of a reporter gene coupled to a single heat shock element

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Abstract Transcription of the heat shock protein Hsp90α was strongly upregulated in human T-cells by interleukin-4 (IL-4) and to a lesser extent by IL-2, reaching peak levels after 2–3 days of stimulation. Heat shock proteins are induced within minutes under stress conditions, via heat shock factors (HSF), which activate heat shock elements (HSE). IL-4, IL-2 and IL-13 upregulated transcription of a reporter gene coupled to a single HSE site and a minimal promoter. HSE may therefore be involved in cytokine induced heat shock gene transcription in the absence of cellular stress.

Key words: Hsp90α; Heat shock factor; Heat shock element; Interleukin-4; Interleukin-2; Interleukin-13

1. Introduction

Heat shock proteins (Hsp) are expressed in increased amounts under stress conditions. They are found in all organisms and are among the most conserved protein families known (for reviews see [1-3]). The protective role of these proteins during stress situations is mainly due to their function as chaperones. Molecular chaperones suppress unspecific aggregation of proteins, and improve yield and kinetics of correct folding during protein synthesis [1,2].

Hsp90 is an abundant, cytosolic protein in eukaryotic cells. The endoplasmic reticulum contains a homologue, Grp94, and corresponding proteins have been described in many organisms, including *Drosophila* (Hsp82) and *Escherichia coli* (HtpG) [1]. Two isoforms, α and β, are encoded by separate genes [2]. In vivo, Hsp90 seems to exist as a dimer. An oligomeric complex between Hsp90, Hsp70 and several other proteins is found in the cytoplasm, which may function as a general chaperone machine [2,3]. Hsp90 binds to unactivated steroid receptors and is required for ligand binding by the receptor [3,4]. It associates with tyrosine kinases of the *src* family, and shuttles these kinases to the membrane, where they are attached by myristoylation [5]. Hsp90 can be coprecipitated with actin and tubulin, suggesting a role in intracellular trafficking [2]. It was proposed that Hsp70, Hsp90 and gp96 form a relay chain for the transfer of peptides to the MHC complex, which implies that they are important for the efficient presentation of antigen [6].

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Abbreviations: CAT, chloramphenicol acetyltransferase; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; HSE, heat shock element; HSF, heat shock factor; Hsp, heat shock protein; PHA, phytohemagglutinin; TK, thymidine kinase of herpes simplex virus.

Heat shock proteins may have additional functions in the immune system. Synthesis of Hsps, including Hsp70 and Hsp90, is upregulated in T-cells by temperatures of 39–41°C, and this upregulation protects the cells against heat-induced suppression of DNA synthesis [7]. Hsps are induced by proinflammatory cytokines: in leukocytes by IL-6 and TNF-α [8], and in oligodendrocytes [9] and myocardial tissue [10] by IL-1. Interferon-α and -β lower the Hsp induction temperature by 1.5°C, without stimulating Hsp synthesis by themselves [11]. Hsp70 and Hsp90 are also upregulated in unstressed cells by the mitogen PHA [12-15] and by the T-cell derived cytokine IL-2 [14].

We have investigated IL-4, an important immunoregulatory cytokine. Mayor functions include the differentiation of T-helper cells into T_H2 cells, which mediate antibody-based immune responses [16], the specific induction of IgE antibodies, which are associated with type I allergic reactions and the defense against helminthic macroparasites [17], and the suppression of proinflammatory cytokines [18,19]. During a differential screening procedure we found that IL-4 upregulates expression of Hsp90α even stronger than IL-2. The upregulation of Hsp90α by both cytokines is slow compared to stress responses, but reporter gene assays suggest that Hsp90α gene expression is activated via a heat shock element (HSE) region.

2. Materials and methods

2.1. Cells and growth factors

PHA-prestimulated peripheral blood lymphocytes and the human erythroleukemia cell line TF-1 [20] were cultured as described, except that GM-CSF was added for TF-1 culture at a concentration of 20 ng/ml [21]. PHA blasts were washed and stimulated for 3 days with 10 ng/ml IL-2 or 50 ng/ml IL-4. Human IL-2, IL-4 and GM-CSF were expressed in *E. coli* and purified as described [22,23]. Recombinant human IL-13 was a generous gift from Dr. N. Vita, Sanofi, Labège, France.

2.2. cDNA library construction

Poly(A)$^+$ RNA was prepared from stimulated cells using oligo(dT)-cellulose (Pharmacia, Quick Prep mRNA Purification Kit). The poly (A)$^+$ RNA was used in the synthesis of *Not*I primer/adapter primed cDNA with the SuperScript cDNA synthesis system (Gibco-BRL Life Technologies) according to the manufacturer's instructions. After addition of *Sal*I adapters, *Not*I digestion, and size selection for fragments >500 bp, the cDNA was ligated directionally into a *Not*I and *Sal*I digested pSPTBM 21 plasmid vector (Boehringer Mannheim), followed by transformation into *E. coli* JM 109 competent cells.

Approximately $3–5 \times 10^5$ recombinants were obtained for each library.

2.3. Differential hybridization screening

Clones were plated at a density of 1000 per 10 cm petri dish, grown overnight at 37°C and cooled to 4°C. Nitrocellulose filter replicas were prepared sequentially from each plate and the DNA was denatured in

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situ and fixed [24]. Prehybridization was performed for 2 h at 42°C in 50% formamide, 6×SSC, 5×Denhardt's solution, 0.5% SDS, 100 μg/ml salmon sperm DNA. Hybridization was performed overnight and was initiated by adding 1×10^6 cpm/ml of ^{32}P-labeled cDNA probe synthesized from poly(A)$^+$ RNA obtained from IL-2 or IL-4 stimulated T-cells. Filters were washed twice in 2×SSC, 0.1% SDS for 5 min at room temperature and in 1×SSC, 0.1% SDS for 30 min at 68°C. After exposure for 24 h at room temperature, colonies that preferentially hybridized with probes derived from IL-4 treated cells were scored and rescreened under the conditions described above.

2.4. RNA blot analysis

Total RNA was isolated by the guanidinium thiocyanate-phenol-chloroform method [25]. The RNA was electrophoresed on a 1.2% formaldehyde gel with 2 μg total RNA per lane and transferred to a positively charged nylon membrane. Following fixation under calibrated UV irradiation, the membranes were hybridized with digoxygenin-labeled probes prepared with a RNA labeling kit (Boehringer Mannheim). The blot was detected using alkaline phosphatase-labeled anti-digoxygenin antibody and a chemiluminescent substrate according to the manufacturer's instructions.

2.5. DNA sequence analysis

Plasmids were isolated from selected clones and vector primers were used to sequence the termini of cDNA inserts using the DyeDeoxy Terminator Cycle sequencing kit (ABI). Further sequence analysis was performed using custom designed oligonucleotide primers. The GenBank and EMBL data bases were searched with the BLAST program [26].

2.6. Reporter gene construction and assays

The promoter/enhancer construct pBLHSE containing a HSE derived from the Hsp90α enhancer region at position −751 to −720 [27] was obtained by cloning a chemically synthesized *SphI/XbaI* fragment between *SphI/XbaI* sites of the CAT vector pBLCAT5 [28]. The following oligonucleotides were used (endonuclease recognition sites and spacer nucleotides are given in lower case):

cttCTTCCGGAAGTTCGGGAGGCTTCTGGAAAAAGcgt
gtacgaaGAAGGCCTTCAAGCCCTCCGAAGACCTTTTTCgcagatc

Plasmids were analyzed by digestion with restriction endonucleases and DNA sequencing, and purified for transfection by cesium chloride density gradients.

2.7. Transient transfection, stimulation and CAT assays

Before transfection, TF-1 cells were cultured in fresh medium without GM-CSF for 24 h. For electroporation, 7×10^6 cells in 350 μl complete medium (supplemented with 5% FCS) were mixed with 30 μg supercoiled plasmid DNA (in 50 μl TE, pH 7.5). The transfection was performed by a single pulse (250 V, 1200 MF, 31–34 ms) from an Eurogentec Easyject pulser. Subsequently, the cells were incubated in 6-well tissue culture plates in complete medium with 20 ng/ml GM-CSF and other cytokines (10 ng/ml) as indicated, for 48 h. For heat shock treatment, after 40 h of culture cells were incubated in a waterbath at 42°C for 30 min, and cultured for another 6 h at 37°C in 5%

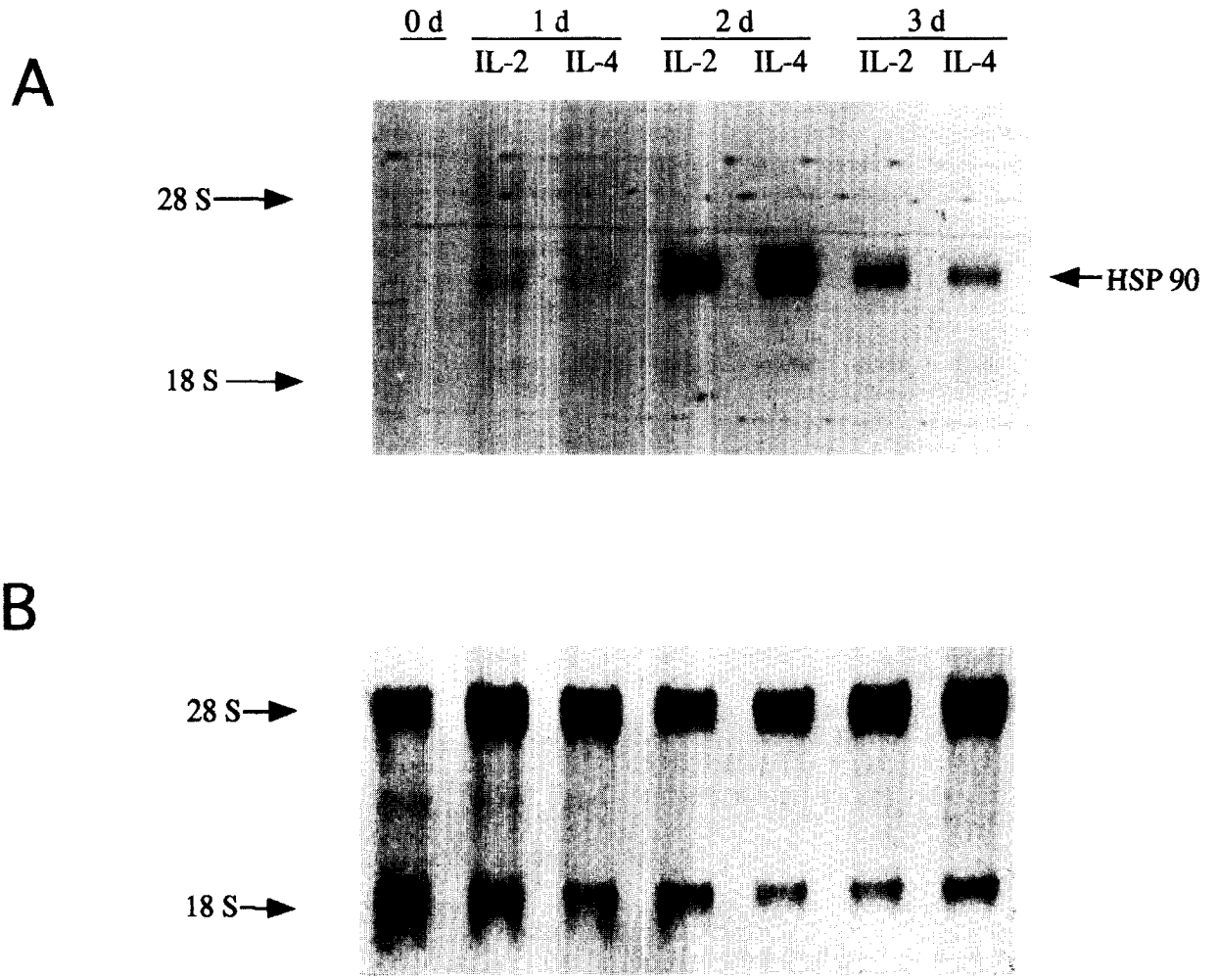

Fig. 1. Kinetics of Hsp90α expression. T-cells were stimulated for the indicated times with IL-2 or IL-4, total RNA was isolated and probed by Northern blotting (A). Loading of comparable amounts of RNA was verified by methylene blue staining (B). Positions of 28S and 18S rRNAs are indicated.

CO_2. Cell extracts were prepared by freeze-thaw procedure. Protein was determined according to Bradford [29] and CAT activities were measured using the CAT ELISA kit from Boehringer Mannheim.

3. Results and discussion

T-cells respond to numerous cytokines, but it is poorly understood to what extent different factors stimulate a distinct differentiation of the activated cells. IL-4 induces a specific cytokine expression profile, leading to the development of T_H2 cells [16]. It stimulates in T-cells the expression of its receptor subunit IL-4Rα [30] and of a triglyceride lipase with homology to pancreatic lipase [31]. In order to identify other IL-4 activated genes, we have produced cDNA libraries from PHA preactivated human T-cells, which were stimulated for 3 days with IL-4 or IL-2 at concentrations saturating for proliferation. Differential screening was used to identify genes preferentially induced by IL-4, but not by IL-2. In the course of this study, a 1000 bp cDNA was obtained which in 4 out of 6 independent experiments hybridized clearly more strongly to mRNA from IL-4 stimulated cells than to mRNA from IL-2 stimulated cells. The cDNA was sequenced and identified as the α isotype of Hsp90.

It was previously shown that IL-2 induces an approximately twofold upregulation of Hsp90α protein after 3–6 h of stimulation [14]. We found strong induction of Hsp90α mRNA by IL-2, and an even more pronounced effect of IL-4. Both cytokines induced maximal mRNA synthesis after 2–3 days of stimulation (Fig. 1).

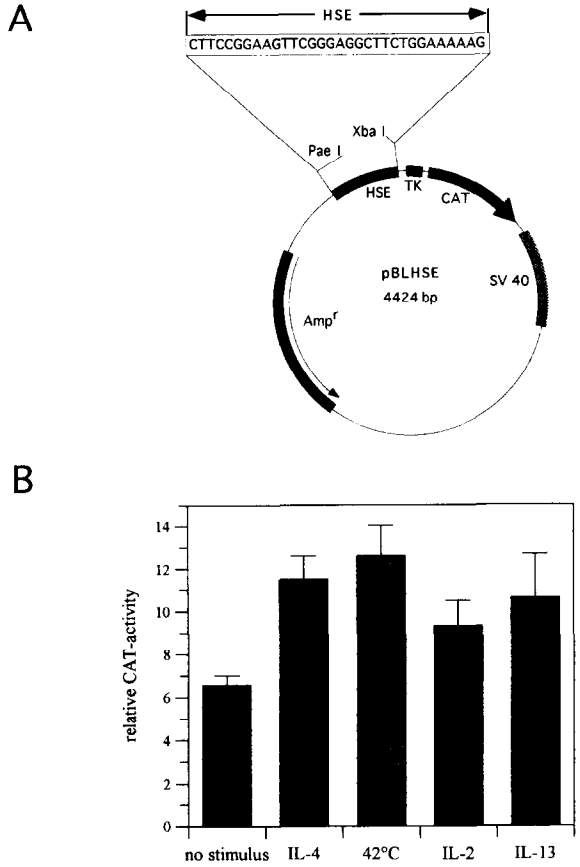

Fig. 2. Transcriptional activation by cytokines and heat induction of the reporter construct pBLHSE, where a single HSE coupled to the TK minimal promoter regulates the CAT gene (A). Expression of CAT was analyzed under various conditions as indicated (B). All values were normalized to the CAT activity of the unstimulated enhancerless pBLCAT5 (=1). Data represent the averaged normalized responses from four representative measurements. Error bars indicate standard error of the mean.

Upregulation of Hsp90α did not correspond to proliferative activity, because IL-4 is a less efficient inducer of proliferation than IL-2 [32,33]. Induction of Hsps in T-cells is not strictly correlated with proliferation, because treatment with PHA, TPA, ionomycin, or ionomycin/TPA combined induces comparable expression of Hsp70 and Hsp90, while PHA stimulates four times higher proliferation than TPA or ionomycin, and two times higher proliferation than both agents combined [12]. The differentiation of U973 cells from a monocytic to a macrophage-like cell type results in growth arrest, but involves elevated transcription of Hsp70 and Hsp90 [34]. Hsp70 is also upregulated during the hemin-induced differentiation of the human erythroleukemia cell line K562 [35]. It is not well understood what functions heat shock proteins have during cell differentiation in the absence of stress. Hsp90 is necessary for the activation of steroid hormone receptors [3] and can also regulate the activity of specific kinases, as shown for v-*src* [5] and for the receptor tyrosine kinase *sevenless* from *Drosophila* [36]. In the fission yeast *Schizosaccharomyces pombe*, a homologue to Hsp90 is required for activation of the tyrosine kinase Wee1, which regulates the length of the G_2 phase by an inhibitory phosphorylation of the Cdc2-cyclin B kinase [37].

The synthesis of heat shock proteins is regulated via promoter elements known as heat shock elements (HSE), which are highly conserved between different promoters [38,39]. HSE bind constitutively expressed heat shock factors (HSF), of which three types have been cloned [40]. Cellular stress induces trimer formation, phosphorylation and binding to HSE.

We have coupled a single HSE derived from the Hsp90α promoter [27] to a TK minimal promoter and tested this construct with a reporter gene assay in the human erythroleukemia cell line TF-1, which proliferates in response to numerous cytokines [20]. The presence of this HSE upregulated transcription approximately fivefold, which may be due to constitutively active transcription factors in this cell line. Omission of GM-CSF from the growth medium reduced the basal transcription rate from pBLHSE only marginally, but impaired cell survival (not shown).

IL-4 significantly upregulated transcription of the reporter gene via the HSE, to approximately the same level as a 30 min heat shock with a temperature of 42°C (Fig. 2). IL-2 and IL-13 induced a weaker but still significant response. This result implicates cytokines in the regulation of gene expression via HSE elements. Such a regulation could either be induced directly via cytokine stimulated signaling pathways, or indirectly via the recruitment of other factors which mediate HSE activation.

The mode of regulation is unclear, in particular with respect to the slow kinetics of Hsp90α upregulation, as HSF can be activated within minutes during heat shock conditions [38]. It has been suggested that in unstressed cells HSF is kept in an inactive state by Hsp70. During cellular stress, misfolded or aggregated proteins appear, which compete with HSF for binding to Hsp70. This releases HSF and allows the assembly of active HSF trimers [39,40]. Other modes of activation clearly exist, because HSF3 does not become activated by stimuli affecting HSF1 or HSF2 [41]. Known inducers of

HSF activity include not only proinflammatory cytokines [8-10], but also the inflammatory mediator arachidonate [42]. IL-4, however, downregulates inflammatory reactions [18,19] and may restrict self-damage and facilitate host repair [43]. The effect of IL-4 on HSE suggests that it may nevertheless lead to activation of HSF by a pathway not identical to the stress response.

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