Mitogen and lymphokine stimulation of heat shock proteins in T lymphocytes

(interleukin 2/heat shock proteins/lymphocytes/stress)

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ABSTRACT We have examined the effects of phytohemagglutinin (PHA) and the polypeptide growth factor interleukin 2 (IL-2) on the synthesis of the 70- and 90-kDa heat shock proteins (HSP70 and HSP90, respectively) in human T lymphocytes. Resting T cells (G₀) stimulated with PHA responded with a generalized increase in protein synthesis that included HSP70. Gel blot analysis indicated that steady-state levels of HSP70 mRNA were not specifically modulated by PHA. Synthesis of HSP90 protein, however, peaked very rapidly following PHA stimulation and decreased sharply after 1 hr. When IL-2-dependent human T cells, synchronized by IL-2 deprivation, were treated with IL-2, synthesis of HSP70 mRNA was increased as much as 15-fold. HSP70 and HSP90 protein synthesis increased significantly upon IL-2 stimulation of human T lymphocytes. Two distinct members of the ancient family of heat shock genes, HSP70 and HSP90, are shown to be stimulated at the activation and progression stages of lymphocyte mitogenesis, which suggests that genetic mechanisms evolved from primitive stress/adaptation responses may be conserved in mammalian cellular activation.

Growth and proliferation are the most fundamental characteristics common to all living organisms, yet the biochemical mechanisms controlling these processes are only vaguely understood. In mammalian T lymphocytes, proliferation requires a minimum of two sequential extracellular signals (1). In vivo the first physiological signal is an antigen for which the cell has a specific clonotypic receptor. The physiological activation of T lymphocytes can be mimicked in situ, however, by nontropic mitogens such as phytohemagglutinin (PHA). The response to antigens or mitogens is characterized by a wave of gene activity, some of which is specific to T cells. Among the T-cell-specific mRNAs induced by PHA are messages for the polypeptide growth factor interleukin 2 (IL-2) and the α chain of the putative IL-2 receptor, or Tac antigen (2, 3). In addition to IL-2 and Tac, other non-T-cell-specific genes are also induced; the most notable genes among these are several nuclear protooncogenes (reviewed in ref. 2). The second extracellular signal required for T-cell proliferation is IL-2, and, once again, the response is characterized by a transient expression of several nuclear protooncogenes and, some 18 hr later, mitosis and cell division (4, 5). These events are well known; however, the steps between the binding of extracellular stimulants, nuclear transcription, and subsequent cell growth are poorly understood.

We have begun to investigate the involvement of a set of ancient genes in the process of mitogen- and lymphokine-mediated cell growth. These genes appear to have evolved from the twin pressures of stress and adaptation.

Heat shock proteins are encoded by a subset of cellular genes known collectively as stress genes. The members of this diverse group can be transcriptionally and posttranscriptionally activated by one or more of a variety of physical or chemical treatments including heat, ethanol, glucose deprivation, sulfhydryl reagents, and heavy metals (see refs. 6–8 for reviews). First described in fruit flies over 20 years ago, remarkably similar proteins have since been found in every phyla of the plant and animal kingdoms in which they have been sought, as well as in bacteria (6). In fact, the 70-kDa heat shock protein (HSP70), which is one of the most prominent heat shock proteins, is among the most highly conserved proteins known, with 48% homology between the Escherichia coli and Drosophila proteins (9). Initially regarded as curiosities, these ubiquitous, highly conserved proteins are now widely believed to serve in vital cellular processes.

We have examined the relationship of mitogen activation and IL-2 stimulation on the synthesis of two major heat shock proteins, HSP70 and the 90-kDa heat shock protein (HSP90), since it is possible that these proteins are involved in both mitogen and IL-2-mediated signaling mechanisms. Here we report that in PHA-treated T lymphocytes HSP90 protein synthesis is very rapidly and transiently increased, while synthesis and accumulation of HSP70 protein progressively increases up to 4 hr after PHA addition. We further show that IL-2 modulates steady-state HSP70 mRNA levels by as much as 15-fold and that HSP70 protein synthesis is increased as a consequence. These studies suggest that genetic elements evolved from primitive cellular stress/adaptation responses are well conserved and may provide vital functions in the growth control of mammalian cells, such as lymphocytes.

MATERIALS AND METHODS

Cells. Fresh human resting peripheral blood T lymphocytes were purified on Ficoll/Hypaque followed by a nylon wool column and Percoll density-gradient centrifugation (10), and T cells (10⁶/ml) were incubated overnight in RPMI 1640 supplemented with 1% glutamine and 5% fetal bovine serum (FBS). IL-2-dependent human T cells were prepared by stimulating fresh T cells with PHA (Wellcome: 1 μg/ml) in RPMI 1640 containing 1% glutamine and 10% FBS for 48 hr. The activated T cells were then washed and incubated 48–72 hr in media containing 200 units of human recombinant IL-2 (Cetus, Emeryville, CA) per ml. Prior to use, the IL-2-dependent human T cells were washed three times and were

Abbreviations: IL-2, interleukin 2; PHA, phytohemagglutinin; HSP70 and HSP90, 70- and 90-kDa heat shock proteins. To whom reprint requests should be addressed.
incubated 24 hr in media containing 1% glutamine and 1% FBS.

**Gel Electrophoresis and Autoradiography.** Lymphocytes were washed twice with methionine-free RPMI 1640 and were resuspended at a concentration of 5 x 10^6/ml. Cells were labeled 1 hr with 0.5 mCi (1 Ci = 37 GBq) of [35S]methionine per ml. After labeling, the cells were washed in 5 ml of cold phosphate-buffered saline (PBS) and lysed in 200 µl of isoelectric focusing sample buffer. Lysates were stored at -70°C. Isoelectric focusing, NaDodSO4/PAGE, and autoradiography were carried out by standard procedures (11).

**Immunoblotting and Immunostaining.** Immunoblotting was accomplished using a Pharmacia-LKB (Gaithersburg, MD) semidry graphite Novablot system. For immunostaining, a 1:50 dilution of monoclonal antibody C92, which is specific for HSP70, was used (12), followed by a 1:1000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG (Kirkegaard and Perry, Gaithersburg, MD). Blocking and washing steps were performed in 50 mM Tris-HCl, pH 7.4/150 mM NaCl/5 mM EDTA/0.25% gelatin.

**Blot Hybridization Analysis.** Resting T cells or growth-arrested IL-2-dependent T cells were stimulated by the addition of PHA (2 µg/ml) or IL-2 (200 units/ml), respectively. Cells were harvested after the indicated period of time, washed once in PBS, and resuspended in guanidium isothiocyanate, and centrifuged on a standard cesium chloride gradient (13).

Equal amounts of total RNA (20 µg) were denatured for 10 min at 55°C in 20 mM morpholinepropanesulfonic acid, pH 7.1/5 mM sodium acetate/1 mM EDTA/50% (vol/vol) deionized formamide (Fluka, Ronkonkoma, NY)/6% formaldehyde, quickly chilled on ice, and size fractionated on 1% agarose gels containing 6% formaldehyde. After the gels were soaked in 20 × SSC (1 × SSC = 150 mM sodium chloride/15 mM sodium citrate, pH 7), RNA was transferred onto nitrocellulose filters (BA 85, Schleicher & Schuell, Keene, NH) by capillary blotting in 20 × SSC using standard procedures (14). Filters were then baked 2 hr under reduced pressure at 80°C.

**Probes and Probe Labeling.** All probes used in this study were purified inserts, which were isolated after appropriate restriction on 1% low-melting-point agarose (Bethesda Research Laboratories). 32P-labeled to 2–5 x 10^8 cpm/µg by primer extension using the PolymeraId labeling kit (PLS) and [35S]dCTP (3000 Ci/mmol, Amersham), and purified on G50 columns (Boehringer Mannheim). The HSP70 probe was a HindIII/Nco I fragment of pH-HSP70(15).

**Hybridization Procedures.** Filters were soaked 2–18 hr in prehybridization buffer (50% formamide/0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin/20 mM Tris, pH 7.4/0.2 mg of salmon sperm DNA per ml/5% dextran sulfate) and hybridized with 10^6 cpm of labeled probe per ml for 24 hr at 42°C. Filters were washed several times in 2 × SSC/0.1% NaDodSO4 at room temperature, washed one to three times for 15 min in 0.1 × SSC/0.1% NaDodSO4 at 50°C, and exposed to Kodak XAR-5 films with Cronex Lightning Plus intensifying screens (E.I. duPont de Nemours) at -70°C for 3–48 hr. Hybrized radioactivity was then removed by immersion in boiling water. Filters were checked prior to use with other probes.

**RESULTS**

**Heat Shock-Induced Biosynthesis of HSP70 and HSP90.** In order to determine which heat shock proteins are predominately induced by hyperthermia, resting T lymphocytes were subjected to a heat shock and pulse labeled with [35S]methionine. Labeled proteins were separated by two-dimensional gel electrophoresis. The results (Fig. 1) indicate that mild heat shock induces the synthesis of two major proteins that migrate with apparent molecular masses of about 70 and 90 kDa. We next tested whether physiological activators of T-lymphocyte proliferation could modulate the expression of heat shock proteins at normal temperature.

**Stimulation of Heat Shock Protein Synthesis by PHA.** Resting peripheral blood T lymphocytes were stimulated with PHA and pulse labeled with [35S]methionine. The labeled proteins were subjected to one-dimensional NaDodSO4 gel electrophoresis, blotted onto nitrocellulose, and immunostained with monoclonal antibody C92, which is specific for HSP70 (12). Densitometric scanning of the autoradiographs indicated that the overall rates of protein synthesis were rapidly elevated by PHA stimulation. Both the rate of HSP70 biosynthesis (Fig. 2B) and the accumulation of HSP70 protein (Fig. 2A and B) increased steadily with similar kinetics during the first through the fourth hour of PHA activation. In contrast, the rate of HSP90 synthesis peaked rapidly and declined steadily from the second through the fourth hours (Fig. 2B). Since a general activator (PHA) of T-lymphocyte competence modulated heat shock protein biosynthesis, we addressed whether the DNA synthesis progression signal stimulated by IL-2 had any effect on heat shock protein expression.

**IL-2-Induced Heat Shock Protein Synthesis.** To determine if IL-2-induced growth might also involve the stimulation of these proteins, we examined the effect of IL-2 treatment on heat shock protein biosynthesis in human IL-2-dependent T lymphocytes. Early G1-synchronized, growth factor-deprived T lymphocytes were allowed to proceed through the cell cycle by the addition of IL-2 and were [35S]methionine pulse labeled at various intervals. Two-dimensional analyses of newly synthesized proteins are shown in Fig. 3. Upon IL-2 stimulation, the rate of HSP70 protein synthesis had more than tripled by the third hour, and by the sixth hour it was still double that in the unstimulated controls. HSP90 synthesis was also induced by IL-2 but with quite different kinetics; no rise in the rate of synthesis was evident until the sixth hour, at which time it was about double that of the control cells (Fig. 3). A control protein (actin) showed little modulation in comparison with HSP70 and HSP90 (data not shown).

**Effect of PHA on HSP70 mRNA in Resting T Lymphocytes.** The capacity of PHA to induce HSP70 protein biosynthesis was further evaluated by assessing mRNA expression. Human resting T lymphocytes were activated by PHA and total mRNAs were purified and subjected to blot hybridization analysis, using an HSP70-specific probe. As shown in Fig. 37°.
**FIG. 2.** PHA stimulates HSP70 and HSP90 synthesis. Resting (G₀ blocked) human T lymphocytes were stimulated with PHA for the indicated times and then pulse labeled with [³⁵S]methionine. The labeled proteins were separated on a 10% NaDodSO₄ gel, transferred to nitrocellulose, immunostained, and autoradiographed. Seventy micrograms of protein was loaded in each lane. Relative synthesis rates were determined from the autoradiograph using an LKB scanning laser densitometer. Relative HSP70 protein accumulation was determined by scanning the immunoblot. (A) Immunostained HSP70 after 0, 1, 2, 3, and 4 hr (left to right) of PHA stimulation. (B) Percent increase in HSP70 and HSP90 synthesis rates and HSP70 protein accumulation after 0–4 hr of PHA stimulation.

4A, the steady-state level of HSP70 message was not modulated by PHA at the time points that we tested. Also shown is the effect of phorbol 12-myristate 13-acetate, which is not able to modulate the level of HSP70 message in those cells. The message for c-myc assessed on the same RNA gel blot showed a 20-fold increase at 4 hr, as expected (Fig. 4B). Consequently, this data suggests that the increase in HSP70 protein following PHA stimulation is due to posttranscriptional regulation and part of a general increase in cellular biosynthesis.

**Effect of IL-2 on Steady-State Levels of HSP70 mRNA.** To assess at which level the synthesis of HSP70 protein was regulated by IL-2, the effect of IL-2 on steady-state levels of HSP70 mRNA was studied. IL-2-dependent human peripheral blood T lymphocytes were blocked in early G₁ by IL-2 deprivation, and total RNA was extracted and subjected to gel blot analysis after various periods of stimulation by IL-2 by using an HSP70-specific probe. Results (Fig. 5) indicate that the steady-state level of HSP70 mRNA is modulated by IL-2; the message is almost undetectable in growth-arrested cells and is induced upon IL-2 stimulation, with a peak of expression around 9 hr. The level of HSP70 message declines upon entry of the cells into S phase (=11 hr after induction). A control probe (18S rRNA) showed no modulation between the different lanes (data not shown).

**DISCUSSION**

T-lymphocyte proliferation requires two sequential signals. Initially, T lymphocytes freshly isolated from peripheral blood are blocked in G₀ and require an activation signal that can be given by either specific antigens or nonspecific polyclonal mitogens such as PHA. The activation signal allows the expression of several gene products, among which are the receptor for IL-2 and IL-2 itself. The binding of IL-2 by high-affinity cell surface receptors transduces a signal that initiates the progression of the activated cells through the cell cycle. Both PHA and IL-2 stimulation cause transient expression of the nuclear protooncogenes c-myc, c-myc, and c-fos; transcription of these genes has often been correlated with the transition to a growing state (reviewed in ref. 3). Although the biological effects of mitogens and IL-2 on T-lymphocyte activation and proliferation are well known, the biochemical events subsequent to mitogen or growth factor binding are unclear.

Recently much attention has been focused on the role of heat shock proteins in cell growth. Two types of evidence suggest that HSP70 is involved at some level in regulating mammalian cell growth. First, there is an intriguing association between HSP70 and two oncogene products as well as the immortalizing protein of a DNA tumor virus. Both c-myc protein (16) and the adenovirus E1A 135 gene product (17, 18) seem to be capable of specifically stimulating expression of the HSP70 gene. More recently it has been shown that a large proportion of the protooncogene-encoded protein p53 was
has been known for some time that HSP90 can be coprecipitated by antibodies directed against pp60 c-src (23), and it has also been shown that HSP90 is sometimes closely associated with steroid receptors (23–25). These findings have led to suggestions that HSP90 may function as a molecular shuttle for a number of important cellular proteins. Naturally, they have also raised questions about how HSP90 might be regulated. One possibility is that HSP90 is regulated by reversible covalent modification, since it is known to be a heavily phosphorylated protein. In fact, a double-stranded DNA-dependent protein kinase activity has been described that phosphorylates HSP90 in vitro; however, it is not clear what effect, if any, phosphorylation or dephosphorylation have on HSP90 (26).

In this study, we have compared the two steps of lymphocyte activation in regard to heat shock protein metabolism. HSP70 protein biosynthesis is specifically induced by the progression signal in quiescent IL-2-deprived T lymphocytes. The regulation seems to occur at the transcriptional level since HSP70 mRNA is also modulated by IL-2. Peak HSP70 steady-state mRNA levels were induced 9 hr after IL-2 treatment, kinetics that are approximately equivalent to those reported for HeLa cells following serum stimulation (21).

In contrast to the apparent transcriptional regulation of HSP70 by IL-2 treatment, the induction of HSP70 mRNA synthesis, protein synthesis, and protein accumulation following PHA stimulation seems to be posttranscriptionally regulated and part of a generalized increase in cellular biosynthetic rates. The kinetics of HSP90 protein synthesis was totally distinct from that of HSP70 after either IL-2 or PHA stimulation. Upon PHA stimulation, HSP90 synthesis rates peak very early and decline rapidly in contrast to HSP70 and most other proteins. Recently it was reported that HSP90 mRNA levels do not change during the cell cycle in HeLa cells (27); therefore, it is possible that the modulations in HSP90 protein synthesis following either PHA or IL-2 are entirely due to posttranscriptional regulation.

Our results suggest that HSP90 synthesis, which is rapidly and transiently induced by PHA, may be more important in the activation of T lymphocytes growth-arrested in G₀. Similarly the specific induction of HSP70 mRNA and protein synthesis by IL-2 indicates a potential role for HSP70 in IL-2-induced cell cycle progression.

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