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Heat Stress Downregulates TCR ζ Chain Expression in Human T Lymphocytes

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Abstract After heat treatment, human T lymphocytes downregulate the T-cell receptor (TCR)/CD3-mediated $[Ca^{2+}]_i$ response and production of inositol triphosphate. Here we demonstrate that heat treatment of T lymphocytes at sublethal temperature decreases the expression of TCR ζ chain, which plays a critical role in the regulation of TCR/CD3-mediated signal transduction. Downregulation of TCR ζ chain in heat-treated T cells was observed at 8 h and reached a maximum at 16 h. Under these conditions, the expression of CD3 ε or TCR αβ chains was minimally affected. Consistent with the decrease in TCR ζ chain, a reduction in the level of TCR/CD3 induced tyrosine phosphorylation of several cellular protein substrates, and a delay in the kinetics of peak tyrosine phosphorylation was observed in heat-treated T cells. Interestingly, analysis of the TCR ζ chain content in the detergent-insoluble membrane fraction showed that heat treatment induces translocation of soluble TCR ζ chain to the cell membranes. In addition, the mRNA level of TCR ζ chain was reduced in heat-treated T cells. Correlative with the downregulation of TCR ζ chain mRNA, the level of the TCR ζ chain transcription factor Elf-1 was also reduced in heat-treated cells. We conclude that heat stress causes a decrease in the level of TCR ζ chain by increasing its association with the membranes and decreasing the transcription of the TCR ζ gene. Decreased expression of the TCR ζ chain is apparently responsible for the decreased TCR/CD3 responses of T cells. J. Cell. Biochem. 79:416–426, 2000.

Key words: heat stress; human T lymphocytes; TCR ζ chain

Exposure of cells to heat stress leads to overexpression of HSPs in the cytoplasm, which subsequently protects the cells from the lethal effects of increased temperatures [Lindquist, 1986; Parsell and Lindquist, 1993; Welch, 1993; Kiang and Tsokos, 1998]. These phylogenetically similar and highly conserved proteins function as molecular chaperones to prevent inappropriate aggregation of proteins and to mediate transport of immature proteins to the target organelles for packaging, degradation, or repair [Beckmann et al., 1990; Parsell et al., 1991]. In addition, HSPs participate in the regulation of the activity of signaling proteins such as steroid receptors [Nathan and Lindquist, 1995] and Ras and Raf kinases [Stancato et al., 1993]. Heat shock proteins serve as substrates in the phosphorylation cascade initiated in response to TCR ligation [Kaur et al., 1989; Ahlers et al., 1994; Egerton et al., 1996] and are involved in antigen presentation [Nossner et al., 1996]. Although the effect of heat exposure on immune function is manifold, heat stress is commonly associated with suppression of immune response [Okimura and Nigo, 1986; Shephard and Shek, 1999].

The mechanism of heat shock–induced immunosuppression remains obscure, but based on T-cell signaling studies, it has been inferred that antigen-induced early signaling events are the targets. In a previous study, we de-

Abbreviations used: HSP, heat shock protein; IP3, inositol triphosphate; ITAM, immunoreceptor tyrosine-based activation motif; PCR, polymerase chain reaction; RT, reverse transcription; TCR, T-cell receptor.

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scribed that heat treating of fresh or cultured human lymphocytes induces a significant downregulation of TCR/CD3-initiated production of IP3 and cytoplasmic free calcium ($[Ca^{2+}]_i$) response [Liossis and Tsokos, 1997]. In addition, we have shown that Jurkat cells overexpressing HSP 70 also display decreased TCR/CD3-mediated early signaling events [Liossis et al., 1997].

The TCR/CD3 is a multisubunit complex consisting of α and β chains, the CD3γ, δ, and ε chains, and a ζ-ζhomodimer or ζ-η or ζ-γ heterodimer [Orloff et al., 1990; Clayton et al., 1991; Weiss and Littman, 1994; Wange and Samelson, 1996]. The disulfide-linked α-β heterodimer is responsible for antigen recognition, whereas CD3εδ, CD3εγ, and ζ-ζcontaining homodimer couple with α-β chains to initiate intracellular signal transduction. The cytoplasmic domain of the TCR ζ chain has three ITAMs, which are necessary for the induction of both proximal and distal events associated with T-cell activation [Irving et al., 1993]. On TCR activation, the tyrosine residues within the ITAMs become phosphorylated, permitting the binding of SH2 domain-containing proteins and subsequently the recruitment of additional signaling molecules. The orderly multistep phosphorylation of the tyrosine residues in these ITAMs are crucial for the coupling of the TCR to various effector functions under different conditions of receptor triggering and for the fidelity of T-cell activation [Zenner et al., 1996; LaFace et al., 1997; Kersh et al., 1998]. The TCR ζ chain also contains a GTP/GDP binding site spanning lysine 128 to glycine 134, which couples GTP binding to TCR ζ chain during signal transduction. [Peter et al., 1992; Sancho et al., 1993]. The TCR ζ chain predominantly binds to the actin cytoskeleton through the third ITAM [Rozdzial et al., 1995; Caplan and Baniyash, 1996] after activation.

The present study was undertaken to gain insight into the molecular mechanism underlying the downregulation of TCR/CD3-mediated cytoplasmic $[Ca^{2+}]_i$ response and the production of IP3 in heat-treated normal T lymphocytes. We demonstrate that heat treatment downregulates TCR ζ chain protein expression in human T lymphocytes. Furthermore, we show that heat treatment-induced downregulation of TCR ζ chain is mediated by an increase in the association of TCR ζ chain with the cytoskeleton and detergent-insoluble membrane fraction. Heat treatment also downregulates the steady-state level of TCR ζ chain mRNA. Accordingly, the TCR/CD3 induced tyrosine phosphorylation of many cellular proteins was reduced in heat-treated cells with a delay in the kinetics. The downregulation of TCR ζ chain, coupled with changes in the molecular distribution, underlies the decreased production of IP3 and intracellular $[Ca^{2+}]_i$ response in heat-treated cells.

MATERIALS AND METHODS

Cells and Antibodies

Monocyte- and macrophage-depleted peripheral blood mononuclear cells (PBMCs) were obtained from the normal donors 18–38 years of age. T lymphocytes were isolated from the PBMCs by depletion of non-T cells using a cocktail of hapten-conjugated antibodies and MicroBeads coupled to anti-hapten monoclonal antibody (mAb) and magnetic separation on MACS column (Miltenyi Biotec, Auburn, CA). In all cases, the percentage purity of T cells in the isolated population is >96% as determined by anti-CD3 ε staining and fluorescence-activated cell sorter (FACS) analysis using an Epics Elite flow cytometer (Coulter, Hialeah, FL). Anti-TCR ζ mAb (clone 6B10.2) and anti-CD3 antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-CD3 mAb, OKT3, was from Ortho Biotech, Inc. (Raritan, NJ). Horseradish peroxidase (HRP)-conjugated antiphosphotyrosine mAb 4G10 was purchased from Upstate Biotechnology Inc. (Lake Placid, NY).

Heat Shock Treatment

Cells were suspended at a concentration of 5×10^6 cells/5 ml in preheated RPMI medium containing 5% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, and were placed in a water bath at 42°C for indicated periods in tissue culture flasks. The cells were then transferred to an incubator and rested at 37°C for various time intervals and harvested for the study. Control T lymphocytes were incubated at 37°C.

Immunoblotting of TCR ζ Chain and Other Signaling Proteins

Five million control or heat-treated T cells in 0.5 ml RPMI-1640 medium were lysed in 1%

Nonidet P-40 (Sigma Chemical Co., St. Louis, MO) lysis buffer as described previously [Liossis et al., 1998]. The protein content of the lysates was determined by using a reagent from Bio-Rad (Hercules, CA). Electrophoretically analyzed proteins (10 μg/lane) were transferred to polyvivyllidene difluoride (PVDF) membranes (Millipore, Bedford, MA), blocked with 3% nonfat dry milk and subsequently immunoblotted with TCR ζ chain, murine mAb 6B10.2, or with the rabbit polyclonal 387 antibody. The blots were incubated with goat anti-mouse HRP-conjugated or with mouse/goat anti-rabbit HRP conjugated antibody, respectively, and developed using the ECL chemiluminescent kit from Amersham Pharmacia Biotec (Piscataway, NJ). Membranes were stripped in Immunopure (Pierce, IL) solution for 1.5 h, reblocked, and reprobed with other antibodies of interest and control mAbs.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis and Immunoblotting of the Detergent-Insoluble Fraction

Five million control or heat-treated T cells in 0.5 ml RPMI-1640 medium were lysed in 1% Nonidet P-40 (Sigma Chemical Co., St. Louis, MO) lysis buffer as described previously [Liossis et al., 1997]. The lysate was centrifuged at 15,000 rpm for 10 min at 4°C. The pellet was solubilized by mechanical agitation in the presence of 4% sodium dodecyl sulfate (SDS) in equal volume of sample loading buffer. The mixture was boiled for 20 min, electrophoretically separated in a 16% gel, and immunoblotted with different antibodies and developed as described above.

RT-PCR Analysis of TCR ζ Chain mRNA Expression

Total RNA was isolated from 5×10^6 control or heat-treated T cells using a RNeasy mini kit (Qiagen, Santa Clarita, CA) according to supplier's directions. Single-stranded cDNA was synthesized using AMV reverse transcriptase–based RT system from Promega (Madison, WI) and oligo-dT primer as instructed by the manufacturer. The primers for human TCR ζ chain were synthesized by Genosys (The Woodlands, TX) and are as follows: TCR ζ chain forward 5′ AGC CTC TGC CTC CCA GCC TCT TTC TGA G 3′ (sense bp 35–62 [Weissman et al., 1988]), reverse 5′ TCA GTG GCT GAG AAG AGT GAA CCG GGT TG 3′ (antisense bp 669–641 [Weissman et al., 1988]). The primers for CD3 ϵ chain are as follows: forward 5′ AAG TAA CAG TCC CAT GAA ACA AAG 3′ (sense bp 31–55) and reverse 5′ TTC TCC AGA GGG TCA GAT GCG TCT CTG 3′ (antisense bp 690–665). The ZAP 70 primers are as follows: forward 5′ GAC GTG GCC ATC AAG GTG CTG AAG CAG 3′ (sense bp 1302–1329) and reverse 5′ GCG CTG CTC CAC GGT CAG GAA GTC G 3′ (antisense bp 1985–1960). RT was carried out in 20 μl, the product was diluted twofold, and 5 μl was used for PCR amplification of TCR ζ chain or diluted fivefold and 5 μl was used for CD3 ϵ chain or ZAP 70 amplification. The amplification was carried out with a Biometra T-3 thermal cycler after initial denaturation at 94°C for 4 min, 33 cycles at 94°C, 45 s; 67°C, 1 min; 72°C for 2 min; and a final extension at 72°C for 7 min. Ten to 15 μl of the PCR products were electrophoresed on 1.2%–1.5% SeaKem agarose gel (FMC BioProducts, Rockland, ME) and visualized with ethidium bromide staining.

Flow Cytometry

The expression of cell surface markers and of TCR ζ chain was examined in partially permeabilized and mildly fixed T cells using a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA) as described previously [Liossis et al., 1998]. Briefly, 10^6 T cells were washed three times in ice-cold phosphate-buffered saline (PBS), and mildly fixed in 0.25% paraformaldehyde for 20 min on ice. Digitonin (Sigma Chemical Co., St. Louis, MO) was added at 10 μg/ml for 5 min on ice, and the efficiency of cell permeabilization was confirmed by the trypan blue exclusion method under a light microscope (always >99%). Cells were incubated with 2 μg of the murine anti-human TCR ζ chain 6B10.2 mAb (or MOPC21 isotypic IgG1 mAb control) for 30 min on ice, after being incubated previously for 30 min on ice with an excess (50×) of human IgG to block nonspecific binding sites. The cells were then washed twice with ice-cold PBS plus 0.05% Tween 20 and further incubated with 4 μl of goat anti-mouse fluorescein isothiocyanate–conjugated antibodies for 30 min on ice. After washing three times with ice-cold PBS/1% fetal bovine serum/0.1% NaN_3, cells were incubated with excess mouse IgG (50×) for 30 min on ice to block the remaining anti-mouse binding sites. Cells were

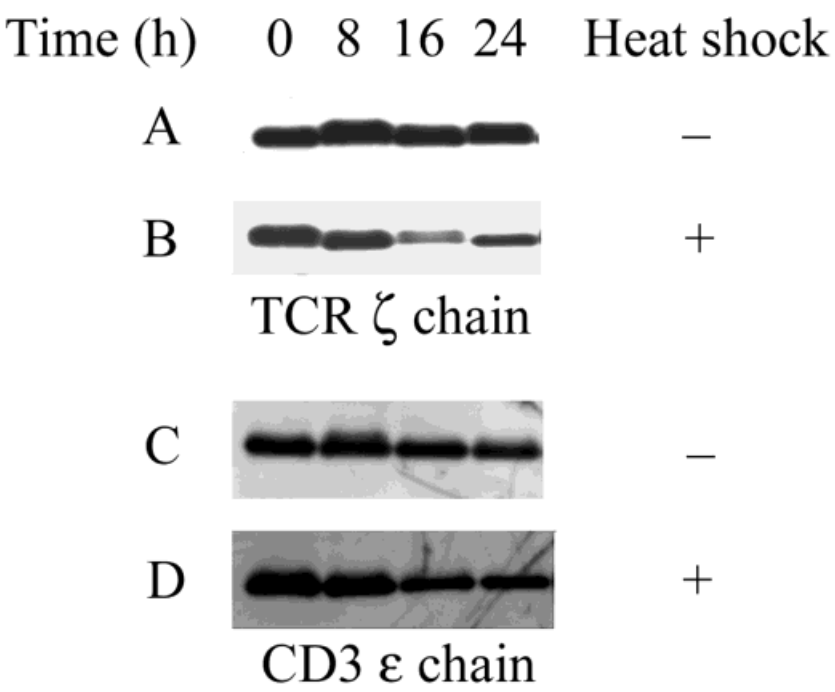

Fig. 1. Heat stress of human T cells causes decreased expression of TCR ζ chain. T cells ($5 \times 10_6$) isolated by negative selection using magnetic beads were treated with or without heat at 42°C for 30 min and incubated at 37°C for 0-, 8-, 16-, and 24-h time points. Cell lysate was prepared with Nonidet P-40 lysis buffer containing various protease inhibitors. Fifteen micrograms of protein lysate was analyzed on 16% SDS-polyacrylamide gel electrophoresis gels under reducing condition and transferred to PVDF membranes. The membrane was immunoblotted with TCR ζ chain mAb 6B10.2 (**A,B**) and CD3 ϵ mAb (**C,D**) and developed using a chemiluminescent kit as described in Materials and Methods.

washed again and fixed in paraformaldehyde (1%) in PBS before analysis. Fixed permeabilized cells were also stained, either with anti-CD3-PE (phycoerythrin) or anti-TCRαβ-PE, to measure the intensity of CD3 ϵ and TCRαβ expression. Results were analyzed using the CellQuest (Becton Dickinson, Rutherford, NJ) analysis software.

Antiphosphotyrosine Immunoblotting

Five million control or heat-shock-treated T cells in 0.5 ml RPMI-1640 medium were stimulated with 10 μg/ml OKT3 for 1, 2, or 3 min at 37°C. The reaction was stopped by the addition of 0.5 ml ice-cold 2× stop buffer (50 mM Tris, 100 mM NaCl, 100 mM NaF, 2 mM Na_3VO_4, 10 mM EDTA, 10 mM sodium pyrophosphate, 2 mM phenylmethyl sulfonyl fluoride (PMSF), 20 μg/ml leupeptin, and 20 μg/ml aprotinin). Cells were pelleted and lysed in 1% Nonidet P-40 (Sigma Chemical Co.) buffer as described previously [Liossis et al., 1998]. Protein content of the lysate was determined by using a reagent from Bio-Rad. Electrophoretically analyzed protein (15 μg/lane) was immunoblotted with the anti-phosphotyrosine–HRP-conjugated 4G10 mAb (1:1,500) and detected using an ECL chemiluminescent kit.

RESULTS

Heat Stress Induces Decreased Expression of TCR ζ Chain in Human T Lymphocytes

We have previously shown that exposure of T cells to heat stress downregulates the TCR/CD3-mediated $[Ca^{2+}]_i$ response and production of IP3 [Liossis and Tsokos, 1997]. TCR ζ chain plays a key role in mediating the biochemical events leading to the production of IP3 and $[Ca^{2+}]_i$ response. To determine whether TCR ζ chain is affected by heat treatment, we measured the TCR ζ chain protein level in T cells after heat treatment at 42°C for 30 min and recovery at 37°C for 8 h, 16 h, and 24 h. Cells were lysed in the presence of NP-40 as described in Materials and Methods. Fifteen micrograms of protein was electrophoresed on 16% SDS-polyacrylamide gel electrophoresis and then transferred to PVDF membranes and

immunoblotted using a TCR ζ chain specific mAb 6B10.2. As shown in Figure 1, the expression of TCR ζ chain started to decrease gradually at 8 h. The decrease in the expression of TCR ζ chain reached maximum after 16 h of post heat-treatment recovery compared to control T cells. At 24 h of recovery, the cells began to reexpress TCR ζ chain, and the level was higher than that seen at 16 h (Fig. 1A,B). Quantitative analysis showed that the level of TCR ζ chain decreased 3%, 36%, 68%, and 52% at 0, 8 h, 16 h, and 24 h in heat-treated cells compared to untreated controls. Western blots of the heat-treated T cells showed a 14-kDa size band that represent either an alternatively spliced or degraded form of TCR ζ chain. This band was decreased at 8 h and was absent from the 16-h samples (Fig. 1B). Immunoblotting experiments using antibody specific to CD3 ϵ chain showed a less severe decrease in the expression of CD3 ϵ chain in heat-treated cells compared to the dramatic decrease found in TCR ζ chain (Fig. 1C,D). The percentage of decrease in the level of CD3 ϵ chain is 4%, 16%, 21%, and 13% at 0 h, 8 h, 16 h, 24 h heat-treated cells compared to untreated controls.

The effect of heat treatment on the expression of TCR ζ chain was also studied by flow cytometry. The cells were treated at 42°C for 30 min and then recovered at 37°C for 16 h. Cells were fixed, permeabilized using digitonin, and stained for TCR ζ chain as described in Materials and Methods. As shown in Figure 2, after 16 h post-treatment recovery, TCR ζ chain staining was decreased compared to the cells incubated at 37°C. Concomitantly, the histogram peak corresponding to unstained cells were increased in heat-treated samples. The percentage of cells in the ζ chain positive population was 76% in controls and 46% in heat-treated cells after 16 h. The percentage of ζ negative population was 22% in controls and 55% in heat-treated cells after 16 h recovery. However, under these conditions, the expression of CD ϵ chain or TCR αβchains was minimally affected in heat-treated samples (Fig. 2). Thus the FACS analysis data were consistent with the decreased expression of TCR ζ chain observed in heat-treated cells by immunoblotting experiments.

TCR ζ Chain Expression in the Detergent-Insoluble Membrane Fraction

It has been shown that 10–40% of the TCR ζ chain is linked to the cytoskeleton in nonactivated T cells [Caplan et al., 1995]. Furthermore, activation of T cells has been shown to cause translocation of the soluble TCR ζ chain to the insoluble membrane fraction. To determine whether the decrease in the level of TCR ζ chain in detergent soluble fraction in heat-treated cells is caused by an increase in the association of TCR ζ chain with the membrane fraction, we analyzed the level of TCR ζ chain in the detergent-insoluble membrane fraction. Concomitant with the decreased expression of TCR ζ chain in the soluble fraction, the level of TCR ζ chain was increased in the detergent-insoluble membrane fraction (Fig. 3A,B). The increase in the level of TCR ζ chain in the detergent-insoluble fraction was seen at 8 h and was highest at 24 h after treatment recovery. Increase in the TCR ζ chain in detergent-insoluble membrane fraction was quantitated by taking a ratio of the levels in heat-treated samples to that of untreated cells at different time points. The result show that in heat-treated cells compared to 0 h, the TCR ζ chain is increased 3.9-, 6.8-, and 22-fold at 8, 16, and 24 h time points, respectively. A significant effect on the translocation of CD3 ϵ chain to detergent-insoluble membrane fraction was not observed in heat-treated cells (Fig. 3C, D). Similarly, heat shock did not change the membrane distribution of another downstream signaling molecule, ZAP 70 (data not shown). Because the TCR ζ chain present in the detergent-insoluble membrane fraction is also associated with actin cytoskeleton [Caplan et al., 1995], we investigated the expression of actin in heat-treated cells to see whether increased expression of actin in the membrane is responsible for more TCR ζ chain binding. As shown in Figure 3E,F, the distribution of the actin in the membrane fraction was similar in heat-treated and untreated cells, suggesting that the translocation from detergent-soluble fraction to insoluble fraction is specific to the TCR ζ chain. These data demonstrate that translocation of the soluble TCR ζ chain to the membrane contributes to the decreased expression of TCR ζ chain in heat-treated cells.

RT-PCR Analysis of TCR ζ Chain mRNA Expression

To investigate whether heat treatment of T cells affects the expression of TCR ζ chain mRNA, we synthesized cDNA from the total RNA isolated from heat-treated and untreated

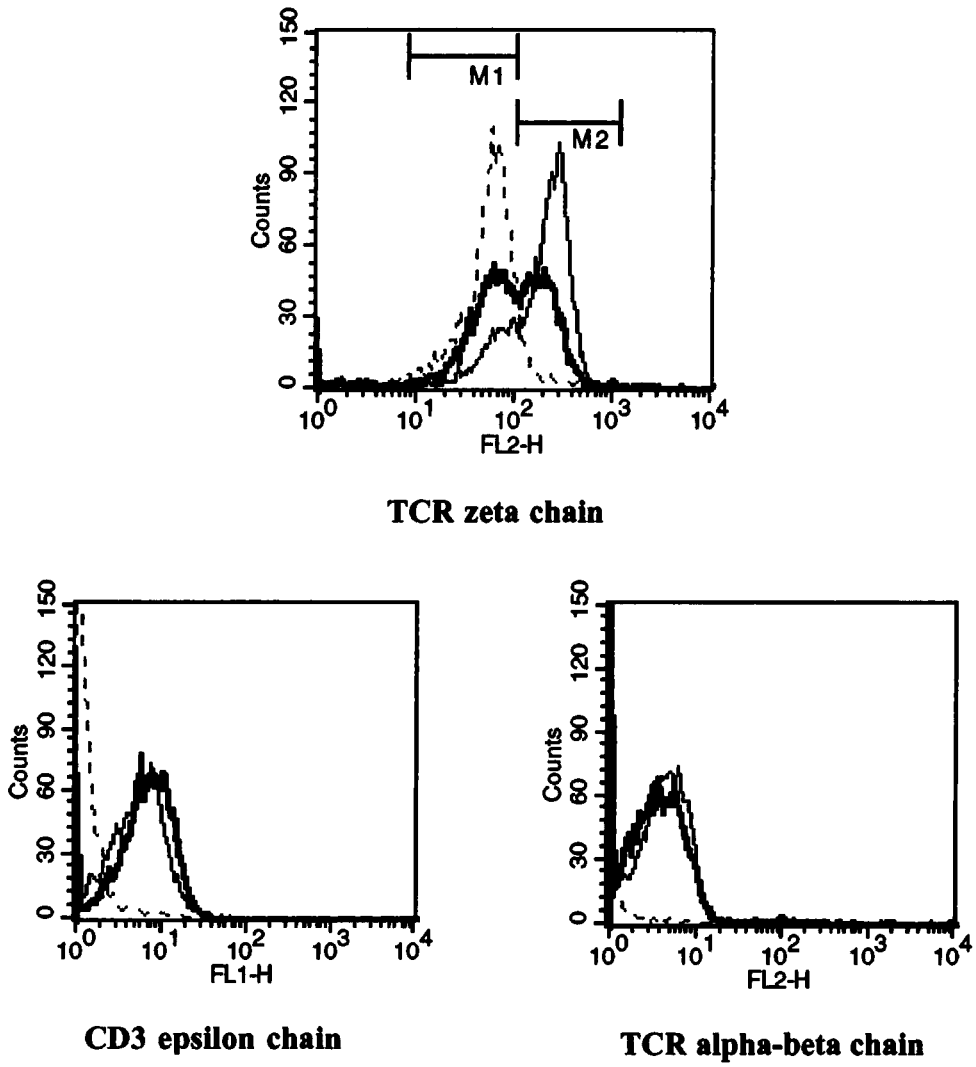

Fig. 2. Flow cytometric analysis of TCR subunits in heat-treated T cells. Magnetically separated T cells (5×10^6) were treated without (control) or with heat at 42°C for 30 min and recovered at 37°C 16 h. Cells were fixed after 16 h and were permeabilized with digitonin and indirectly stained with a murine anti-TCR ζ chain mAb and PE-conjugated goat anti-mouse antibody as described in Materials and Methods (negative control: broken line; control: thin line; heat-treated cells: thick line). Permeabilized cells were also labeled with PE-conjugated mouse anti-CD3 mAb or TCR αβ polyclonal antibody. The staining of TCR ζ chain was significantly decreased in heat-treated cells, whereas CD3 ϵ or TCR αβ chains were minimally affected. In TCR ζ staining, the percentage of cells were M1: 76%, M2: 22% in controls, and M1: 46%, M2: 55% in heat-treated cells.

cells at different time points of recovery. The cDNA was PCR amplified by a pair of TCR ζ chain-specific primers as described in Materials and Methods. As shown in Figure 4A, agarose gel electrophoresis of the RT-PCR products of TCR ζ chain showed a band migrating at the expected size of 635 bp in all of the heat-treated samples. The intensity of the PCR product remained similar in control, untreated samples at 0, 8, 16, and 24 h (Fig. 4A). In heat-treated samples, a major decrease in the TCR ζ chain mRNA was observed at 8 h after heat treatment recovery (Fig. 4A). The decrease was highest at 16 h and the TCR ζ chain mRNA level began to gradually increase at 24 h. The changes in the level of TCR ζ chain mRNA paralleled those observed for TCR ζ chain protein (Fig. 1B and Fig. 4A). The expression of CD3 ε chain was not significantly affected under these conditions (Fig. 4B). RT-PCR analysis of ZAP-70, a downstream protein involved in TCR/CD3-mediated signal trans-

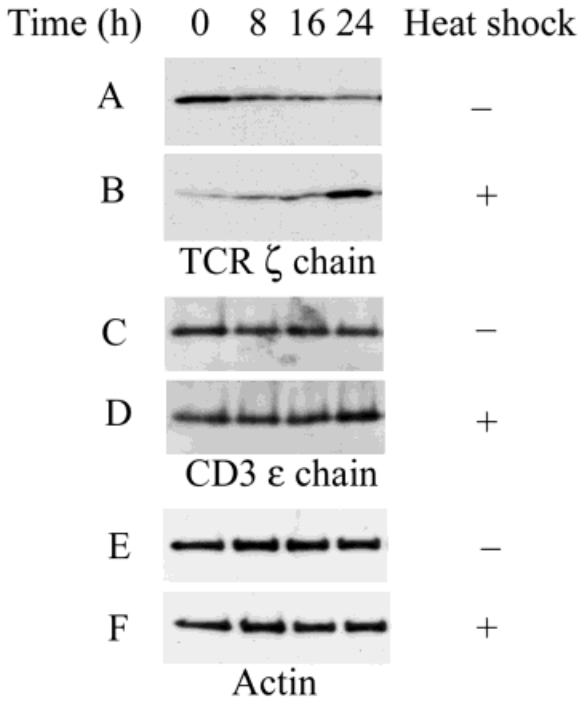

Fig. 3. Heat stress of human T cells causes increased expression of TCR ζ chain in detergent-insoluble membrane fraction. T cells (5×10^6) isolated by magnetic beads were treated without (control) or with heat at 42°C for 30 min and incubated at 37°C for 0-, 8-, 16-, and 24-h time points. Cell lysate was prepared with Nonidet P-40 lysis buffer containing various protease inhibitors and centrifuged at 15,000 rpm to separate the supernatant. The insoluble membrane pellet was solubilized by mechanical agitation in 4% SDS and boiling for 15 min as described by Caplan et al. [1995]. The solubilized membrane fraction was analyzed on 16% SDS–polyacrylamide gel electrophoresis gels under reducing condition and transferred to PVDF membranes. Western blotting was carried out with TCR ζ chain mAb 6B10.2 (**A,B**), anti-CD3 ϵ chain (**C,D**) and anti-actin antibodies (**E,F**). The immunoblots were developed with HRP-conjugated secondary antibodies and chemiluminescent detection as described in Materials and Methods. The level of TCR ζ chain was increased in the detergent-insoluble membrane fraction of heat-treated cells.

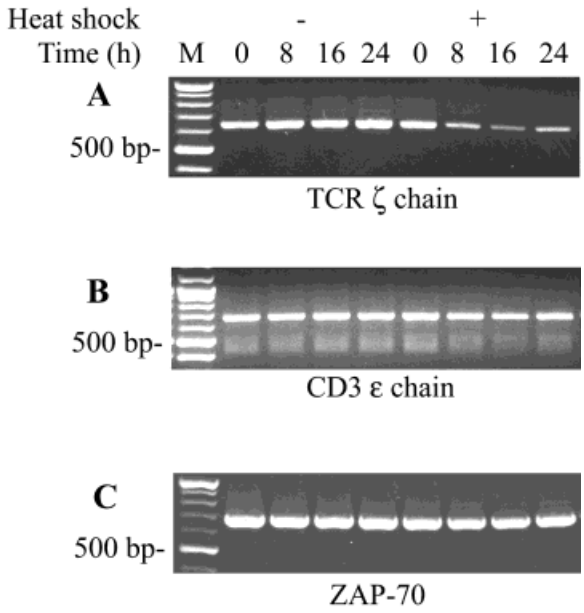

Fig. 4. RT-PCR analysis of TCR ζ chain mRNA expression in heat-treated T cells. T cells (5×10^6) isolated using magnetic beads were treated without or with heat at 42°C for 30 min and incubated at 37°C for 0-, 8-, 16-, and 24-h time points. Total RNA was isolated from the T cells and then reverse transcribed using AMV reverse transcriptase. cDNA was diluted and TCR ζ chain (**A**), CD3 ϵ chain (**B**), and ZAP 70 (**C**) were PCR amplified by using specific primer pairs as described in Materials and Methods. Ten μl of the PCR product was electrophoresed on 1.2% agarose gels and stained with ethidium bromide. M, 100-bp molecular weight ladder.

duction, also showed no major differences in the level of mRNA expression in the presence or absence of heat treatment (Fig. 4C). Thus, heat treatment specifically decreases TCR ζ chain mRNA expression in human T cells.

Expression of the TCR ζ Chain Transcription Factor Elf-1 in Heat-Treated Cells

TCR ζ chain has two binding sites for transcription factor Elf-1, which is involved in the transcriptional regulation of TCR ζ chain [Rellahan et al., 1998]. To investigate whether the decrease in the TCR ζ chain mRNA in heat-treated cells is the consequence of a decrease in the expression of the transcription factor Elf-1, we performed Western blot analysis using an antibody specific to Elf-1. The result of the immunoblotting experiment is shown in Figure 5. Heat treatment significantly reduced the levels of both the 95-kDa and 70-kDa forms of the Elf-1 in T cells. The decrease in the 95-kDa form was observed at 30 min after heat treatment and was nearly absent at 16 h and 24 h after recovery samples (Fig. 5A,B). In contrast, the detergent-insoluble fraction showed an increase in the 95-kDa form of Elf-1 in heat-treated samples (Fig. 5D). The ratio of the Elf-1 in the detergent-insoluble fraction to that of soluble fraction was 3, 3, 42, and 62 at 0-, 8-, 16-, and 24-h time points, respectively. A marginal increase in the 95-kDa form of Elf-1 was also found in the untreated control T cells (Fig. 5C). The ratio of the Elf-1 in the detergent-insoluble fraction to that of the soluble fraction in untreated cells was 0.3, 0.6, 1, and 1.5 at 0-, 8-, 16-, and 24-h time points. These data sug-

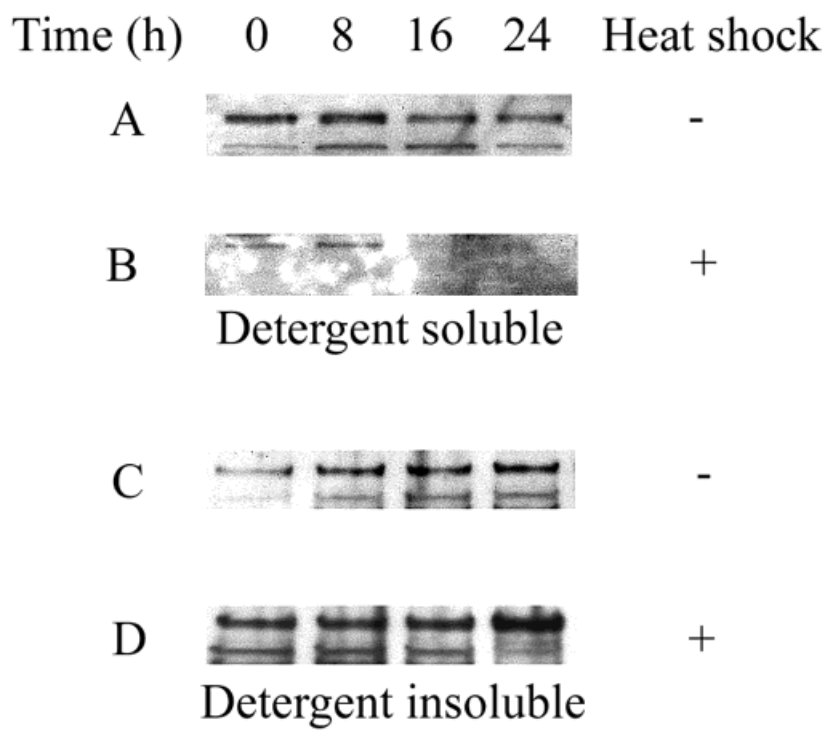

Fig. 5. Expression of TCR ζ chain transcription factor Elf-1 in the detergent-soluble and -insoluble fraction of T cells. T cells (5×10^6) isolated using magnetic beads were treated with or without heat for 30 min and incubated at 37°C for 0-, 8-, 16-, and 24-h time points. Cell lysate was prepared with Nonidet P-40 lysis buffer containing various protease inhibitors. The cell lysate was centrifuged at 15,000 rpm for 10 min at 4°C. After separating the supernatant, the pelleted membrane fraction was solubilized by 4% SDS and boiling for 15 min in lysis buffer. Fifteen micrograms of protein lysate or the solubilized membrane fraction was analyzed on 16% SDS–polyacrylamide gel electrophoresis gels under reducing conditions and transferred to PVDF membranes. The membrane was immunoblotted with Elf-1 antibody and developed using ECL chemiluminescent kit as described in Materials and Methods. **A,B:** Detergent-soluble fraction. **C,D:** Detergent-insoluble membrane fractions.

gest that heat stress causes decreased expression of TCR ζ chain mRNA by decreasing the level of Elf-1, which is the main transcription factor of the TCR ζ chain gene.

Heat Treatment Downregulates TCR/CD3-Induced Phosphorylation

The first step in the signal transduction after TCR/CD3 engagement is the tyrosine phosphorylation of several cellular substrates. To determine whether decreased expression of TCR ζ chain alters the TCR/CD3-mediated tyrosine phosphorylation of cellular substrates, we stimulated heat-treated and untreated cells after 16 h recovery, with an anti-CD3 specific antibody, OKT3, for 1, 2, and 3 min. As shown in Figure 6, the OKT3 induced phosphorylation of cellular substrates was significantly decreased in heat-treated cells. Interestingly, a protein migrating at an apparent molecular weight of 38 kDa was noted to increase in phosphorylation. It is quite possible that this may represent the p38 stress mitogen-activated protein (MAP) kinase, which is activated in heat-treated cells [Naguyen and Shiozaki, 1999]. Consistent with the decreased expression of TCR ζ chain protein, the tyrosine phosphorylation of the corresponding p21 band was also low in heat-treated cells compared to untreated controls (Fig. 6). However, low levels of the p23 form of phosphorylated TCR ζ chain was found in heat-treated cells. The kinetics of tyrosine phosphorylation of cellular proteins in control cells is similar to our previous results [Liossis et al., 1998] with a peak at 2 min of OKT3 stimulation. In contrast, the kinetics of tyrosine phosphorylation of cellular substrates is delayed in heat-treated cells with a peak after 2 min (Fig. 6).

DISCUSSION

Our previous observation that exposure of T lymphocytes to heat stress decreased CR/CD3-

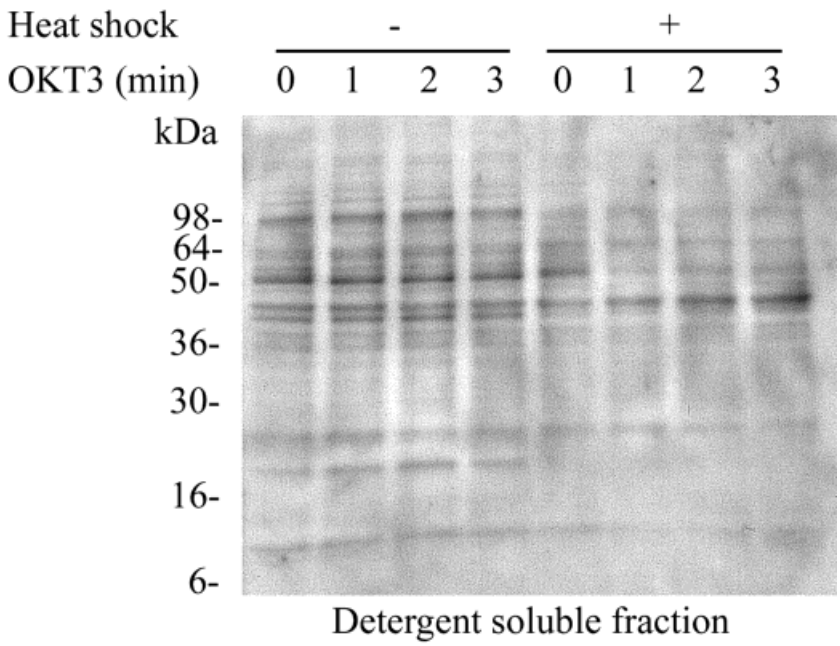

Fig. 6. TCR/CD3-induced tyrosine phosphorylation of cellular substrates in heat-treated cells. T cells (5×10^6) were isolated by magnetic separation and treated with or without heat for 30 min and incubated at 37°C 16 h. Cells were then stimulated with 10 μg OKT3 for 0, 1, 2, and 3 min. Stop buffer was added, and the cells were pelleted and lysed with Nonidet P-40 lysis buffer containing various protease inhibitors. Fifteen micrograms of protein lysate was analyzed on 16% SDS–polyacrylamide gel electrophoresis gels under reducing conditions and transferred to PVDF membranes. The membrane was immunoblotted with HRP-conjugated antiphosphotyrosine antibody 4G10 and developed using an ECL chemiluminescent kit as described in Materials and Methods. Molecular sizes (kDa) are shown on the left.

induced $[Ca^{2+}]_i$ responses and IP3 production led us to hypothesize that heat stress altered the expression of one or more signaling molecules. Indeed, the results presented herein clearly show that heat stress caused decreased expression of the TCR ζ chain. We found that heat stress causes increased translocation of the TCR ζ chain to the detergent-insoluble membrane fraction of the cell, whereas it causes decreased expression of the main TCR ζ chain transcription factor, Elf-1. Apparently, the decreased level of Elf-1 could be responsible for the decreased level of TCR ζ chain mRNA in heat-treated cells. The decrease in the level of Elf-1 seems to be the consequence of its translocation to the detergent-insoluble membrane fraction (Fig. 5), possibly the nuclear membranes. Although reduced in amount, translocation of Elf-1 was found in control T cells incubated at 37°C, suggesting that this process occurs under physiological conditions and that heat treatment enhances it. Because of the presence of 95-kDa and 70-kDa forms, it has been suggested that Elf-1 undergoes posttranslational modifications, and it will be interesting to study whether changes in these modifications cause the translocation of Elf-1 to the membrane after its transport to the nucleus.

It has been shown that 10–40% of the TCR ζ chain is associated with the cell membrane [Caplan et al., 1995; Caplan and Baniyash, 1996]. In addition, T-cell activation causes tyrosine phosphorylation of TCR ζ chain and the phosphorylated form binds to the actin cytoskeleton and thus translocates to the membrane [Caplan et al., 1995; Rozdzial et al., 1998]. Although our results suggest that heat treatment causes mobilization of the soluble TCR ζ chain to the membrane, the exact mechanism involved in the increased association of TCR ζ chain with the membrane fraction is unclear. It is possible that the changes in the organization of the actin cytoskeleton induced by heat treatment may initiate this process. It has been shown that hsp27 limits actin stress fiber and focal adhesion formation after heat shock [Schneider et al., 1998]. Depolymerization of the actin cytoskeleton may trap the TCR ζ chain in the membrane fraction. In support of this hypothesis, Rozdzial et al. [1995] have

shown that the microfilament poison cytochalasin D enhances the coprecipitation of actin with TCR ζ chain after receptor ligation. Another possible reason for the decrease of TCR ζ chain in the soluble pool is that heat treatment may activate T cells, leading to the phosphorylation of TCR ζ chain and its translocation to membranes or ubiquitin-mediated degradation [Valitutti et al., 1997]. It has been shown that partially phosphorylated TCR ζ chain migrates at 21 kDa and the totally phosphorylated form migrates at 23 kDa [Kersh et al., 1998]. The absence of the 21-kDa phosphorylated form and the presence of the 23-kDa phosphorylated form in heat-treated cells suggests that heat treatment directly or indirectly induces rapid total phosphorylation of the available ITAM tyrosines rather than the hierarchical phosphorylation observed under antigenic stimulation. Because phosphorylation of TCR ζ chain activates its translocation to membrane [Caplan and Baniyash, 1996; Rozdzial et al., 1995, 1998], it is conceivable that total phosphorylation of the TCR ζ chain may strongly drive its translocation to membranes. Conversely, activation of JNK or p38 stress MAP kinases followed by heat shock [Naguyen and Shiozaki, 1999] may enhance the phosphorylation of TCR ζ chain and its subsequent association with the membrane. Involvement of other kinases in addition to Lck and Fyn in the phosphorylation and cytoskeletal association of TCR ζ chain has been described previously [Rozdzial et al., 1998].

Deletion of exon VII of TCR ζ chain results in a putative protein of 14-kDa lacking the third ITAM that is predominantly involved in actin cytoskeleton binding. We identified a 14-kDa form of the TCR ζ chain at time points 0 and 8 h in heat-treated cells (Fig. 1). We suspect that this is an alternatively spliced TCR ζ chain. It is possible that heat stress may cause splicing aberrations, and the resulting protein product may undergo rapid degradation.

Consistent with the decrease in the TCR ζ chain, the TCR/CD3-induced tyrosine phosphorylation of cellular substrates was dramatically reduced in heat-treated cells. The decrease in the tyrosine phosphorylation of cellular substrates was followed by a delay in the peak phosphorylation kinetics in heat-treated cells. It has been proposed that TCR triggering is a consequence of the redistribution of TCR into an environment enriched in tyrosine kinases and depleted of tyrosine phosphatases. The TCR is associated with lipid rafts, and cross-linking of the TCR causes aggregation of raft-associated proteins [Janes et al., 2000]. Raft aggregation promotes tyrosine phosphorylation and recruitment of signaling proteins and excludes tyrosine phosphatase CD45 [Janes et al., 2000]. It is possible that T cells at high temperature inhibit lipid raft formation by altering the dynamics of membrane lipids. The inhibition of raft formation could lead to association of CD45 phosphatase with TCR and dephosphorylation of recruiting signaling proteins. The decrease in TCR/CD3-induced tyrosine phosphorylation will explain the observed downregulation of TCR-mediated $[Ca^{2+}]_i$ responses and IP3 production in heat-treated cells. Thus, the picture emerging from our result is that, heat stress suppresses the antigen receptor-initiated T cell response by decreasing the level of TCR ζ chain. The decrease in the TCR ζ chain is due to decreased transcription of the TCR ζ gene and increased translocation to the detergent insoluble fraction of the cells.

REFERENCES

Ahlers AK, Engel C, Scott M. Gaestel F, Herrmann F, Brach MA. 1994. Interleukin 3 and granulocyte colony stimulating factor induce activation of the MAPKAP kinase 2 resulting in in vitro serine phosphorylation of the small heat shock protein (HSP 27). Blood 83:1791–1796.

Beckmann RP, Mizzen LE, Welch WJ. 1990. Interaction of hsp-70 with newly synthesized proteins: implications for protein folding and assembly. Science 248: 850–853.

Bolliger L, Johansson B, Palmer E. 1997. The extracellular domain of the T cell receptor zeta chain is involved in assembly and signal transduction. Mol Immunol 34: 819–827.

Caplan S, Baniyash M. 1996. Normal T cells express two T cell antigen receptor populations, one of which is linked to the cytoskeleton via ζ chain and displays a unique activation-dependent phosphorylation pattern. J Biol Chem 271:20705–20712.

Caplan S, Zeliger S, Wang L, Baniyash M. 1995. Cell-surface expressed T-cell antigen receptor ζ chain is associated with the cytoskeleton. Proc Natl Acad Sci USA 92:4768–4772.

Clayton LK, D'Adamio LD, Howard FD, Seih M, Hussey RE, Koyasu S, Reinherz EL. 1991. CD3η and CD3ζ are alternatively spliced products of a common genetic locus and are transcriptionally and/or post-transcriptionally regulated during T cell development. Proc Natl Acad Sci USA 88:5202–5206.

Egerton M, Moritz RL, Druker B, Kelso A, Simpson RJ. 1996. Identification of the 70 kDa heat shock cognate protein (Hsc70) and alpha-actinin-1 as novel phosphotyrosine containing proteins in T lymphocytes. Biochem Biophys Res Commun 224:666–674.

Irving BA, Chan AC, Weiss A. 1993. Functional characterization of a signal transducing motif present in the T cell antigen receptor ζ chain. J Exp Med 177:1093–1103.

Janes PW, Ley SC, Magee AI, Kabouridis PS. 2000. The role of lipid rafts in T cell antigen receptor (TCR) signaling. Semin Immunol 12:23–34.

Kaur P, Welch WJ, Saklatvala J. 1989. Interleukin 1 and tumor necrosis factor increase phosphorylation of the small heat shock protein: effects in fibroblasts, HepG2, and U937 cells. FEBS Lett 258:269–271.

Kersh EN, Shaw AS, Allen PA. 1998. Fidelity of T cell activation through multistep T cell receptor ζ phosphorylation. Science 281:572–575.

Kiang JG, Tsokos GC. 1998. Heat shock protein 70 kDa: molecular biology, biochemistry and physiology. Pharmacol Ther 80:183–201.

La Face DM, Couture C, Anderson K, Shih G, Alexander J, Setto A, Mustelin T, Altmaan A, Grey HM. 1997. Differential T cell signaling induced by antagonist peptide-MHC complexes and the associated phenotypic responses. J Immunol 158:2957–2064.

Lindquist S. 1986 The heat shock response. Annu Rev Biochem 55:1151–1191.

Liossis SNC, Tsokos GC. 1997. Heat shock of normal T-cells and cell lines downregulates the TCR/CD3-mediated cytoplasmic Ca^{2+} responses and the production of inositol triphosphate. Immunopharmacol Immunotoxicol 19:511–521.

Liossis SNC, Ding XZ, Kiang JG, Tsokos GC. 1997. Overexpression of the heat shock protein 70 enhances the TCR/CD3- and Fas/Apo-1/CD95-mediated apoptotic cell death in Jurkat cells. J Immunol 158:5668–5675.

Liossis SNC, Ding XZ, Dennis GJ, Tsokos GC. 1998. Altered pattern of TCR/CD3-mediated protein tyrosyl phosphorylation in T cells from patients with systemic lupus erythematosus. J Clin Invest 101:1448–1457.

Nathan DF, Lindquist S. 1995. Mutational analysis of HSP-70 function: interactions with a steroid receptor and a protein kinase. Mol Cell Biol 15:3917–3925.

Nguyen AN, Shiozaki K. 1999. Heat-shock-induced activation of stress MAP kinase is regulated by threonine- and tyrosine-specific phosphatases. Genes Dev 13:1653–1663.

Nossner E, Goldberg JE, Naftzger C, Lyu SC, Berger C, Krensky AM. 1996. HLA-derived peptides which inhibit T cell function bind to members of the heat shock protein 70 family. J Exp Med 183:339–348.

Okimura T, Nigo Y. 1986. Stress and immune responses: suppression of T cell function in restraint stressed mice. Jpn J Pharmacol 40:505–511.

Orloff DG, Ra C, Frank SJ, Klausner RD, Kinet JP. 1990. Family of disulphide dimers containing ζ and η chains of the T cell receptor and γ chain of Fc receptors. Nature 347:189–191.

Parsell DA, Lindquist S. 1993. The function of heat shock proteins in stress tolerance: degradation and reactivation of damaged proteins. Annu Rev Genet 27:437–496.

Parsell DA, Sanchez Y, Stitzel JD, Lindquist S. 1991. Hsp104 is a highly conserved protein with two essential nucleotide-binding sites. Nature 353:270.

Peter ME, Hall C, Ruhlmann A, Sancho J, Terhorst C. 1992. The T cell receptor ζ-chain contains a GTP/GDP binding site. EMBO J 11:933–941.

Rellahan BL, Jensen JP, Howcroft HK, Singer DS, Bonvini E, Weissmann AM. 1998. Elf-1 regulates basal expression from the T cell antigen receptor ζ-chain promoter. J Immunol 160:2794–2801.

Rozdzial MM, Malissen B, Finkel TH. 1995. Tyrosine phosphorylated T cell receptor zeta chain associates with actin cytoskeleton upon activation of mature T lymphocytes. Immunity 3:6234–6233.

Rozdzial MM, Pleiman CM, Cambier JC, Finkel TH. 1998. $pp56^{LCK}$ mediates TCR ζ chain binding to the microfilament cytoskeleton. J Immunol 161:5491–5499.

Sancho J, Peter ME, Franco R, Danielian S, Kang JS, Fagard R, Woods J, Reed JC, Kamoun M, Terhorst C. 1993. Coupling of GTP-binding to the T cell receptor (TCR) ζ-chain with TCR mediated signal transduction. J Immunol 150: 3230–3242.

Schneider GB, Hamno H, Cooper LF. 1998. In vivo evaluation of hsp27 as an inhibitor of actin polymerization: hsp 27 limits actin stress fiber and focal adhesion formation after heat shock. J Cell Physiol 177:575–584.

Shephard RJ, Shek PN. 1999. Immune dysfunction as a factor in heat illness. Crit Rev Immunol 19:285–302.

Stancato LF, Chow YH, Hutchison KA, Perdew GH, Jove R, Pratt WB. 1993. Raf exists in a native heterocomplex with hsp70 and p50 that can be reconstituted in a cell free system. J Biol Chem 268:21711–21716.

Valitutti S, Muller S, Salio M, Lanzavecchia A. 1997. Degradation of T cell receptor (TCR)-CD3-ζcomplexes after antigenic stimulation. J Exp Med 185:1859–1864.

Wange RL, Samelson LE. 1996. Complex complexes: signaling at the TCR. Immunity 5:197–205.

Weiss A, Littman DR. 1994. Signal transduction by lymphocyte antigen receptors. Cell 76:263–274.

Weissman AM, Hou D, Orloff DG, Modi WS, Seuanez H, O' Brien SJ, Klausner RD. 1988. Molecular cloning and chromosomal localization of the human T-cell receptor ζ chain: distinction from the molecular CD3 complex. Proc Natl Acad Sci USA 85:9709–9713.

Welch WJ. 1993. Heat shock proteins functioning as molecular chaperons: their roles in normal and stressed cells. Philos Trans Soc Lond Biol 339:327–333.

Zenner G, Vorherr T, Mustelin T, Burn P. 1996. Differential and multiple binding of signal transducing molecules to the ITAMS of the TCR ζ chain. J Cell Biochem 63:94–103.