Sequestration of NF-κB Signaling Complexes in Lipid Rafts Contributes to Repression of NF-κB in T Lymphocytes under Hyperthermia*

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Sepsis causes extensive apoptosis of lymphocytes, a pathological condition that is frequently associated with hyperthermia. Heat stress has been implicated to repress the activation of an inflammatory mediator, nuclear factor of κB (NF-κB), which sensitizes cells to apoptosis mediated by inflammatory cytokine, tumor necrosis factor α. However, the molecular mechanism of hyperthermia-associated loss of T cells remains unclear. We show that hyperthermia causes rapid translocation of IκB kinase (IKK) and NF-κB complexes into the plasma membrane-associated lipid rafts in T cells. Heat stress induces aggregation of Carma1 in lipid rafts, which in turn recruits protein kinase Cθ (PKCθ) and Bcl10 to the microdomains, causing subsequent membrane translocation of the IKK and NF-κB signalosomes. Depletion of Carma1 and inhibition of PKCθ impair accumulation of NF-κB complexes in lipid rafts. Heat stress prohibits IκB kinase activity by sequestering the IKK and NF-κB complexes in lipid rafts and by segregating the chaperone protein Hsp90, an essential cofactor for IKK, from the IKK complex. This process ultimately results in functional deficiency of NF-κB and render T cells resistant to tumor necrosis factor α-induced activation of IKK, thereby contributing to the apoptotic loss of T lymphocytes in sepsis-associated hyperthermia.

Bacterial endotoxin in sepsis stimulates immune cells to produce abundant pro-inflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukine-1 (IL-1). As the consequence of hyper-inflammation, severe cell and organ injury may occur. One of the characteristic features of sepsis is extensive apoptosis of lymphocytes and gastrointestinal epithelial cells, which is often associated with rising body temperature or hyperthermia. In response to heat stress, Hsp70, a stress-induced chaperone of the Hsp70 family, is markedly elevated to execute cytoprotective functions by rejuvenating damaged and aggregated intracellular proteins. Aside from the chaperone function, Hsp70 has been shown to inhibit apoptosis by interfering with the assembly of Apaf-1 apoptosome and by antagonizing the activity of apoptosis-inducing factor. Contrary to the anti-apoptotic effect of Hsp70, other studies showed that the stress-induced Hsp70 suppresses cell proliferation and sensitizes cells to apoptosis induced by TNFα, FasL, and TRAIL (TNF-related apoptosis-inducing ligand). During sepsis, TNFα or endotoxin activates NF-κB, one of the key mediators of inflammation, whereas overexpression of Hsp70 during heat stress was reported to repress the activity of NF-κB through binding to IκκB, a regulatory subunit of IκB kinase (IKK) complex, in promoting apoptosis in certain types of adhesive cell lines. Yet, it remains unclear for the molecular mechanism of sepsis/hyperthermia-related loss of lymphocytes, and it is also unknown about the fate of IKK in the early response to hyperthermia before Hsp70 is synthesized.

The transcriptional factor NF-κB, including multiple subunits consisting of RelA, RelB, c-Rel, p50/p105, and p52/p100, has prominent roles in immune and inflammatory responses (for review, see Refs. 12–14). In resting T cells, NF-κB is sequestered in the cytoplasm in complex with its inhibitor, IκB. Upon ligation of T cell receptor (TCR), sequential activation of upstream kinases and signaling molecules transmits the activation signal to the IKK complex, which comprises two catalytic subunits, Iκκα and IκkB, and one regulatory subunit, IκκB. Activated Iκκα and IκkB in turn serine-phosphorylates IκB, resulting in ubiquitination and proteasomal degradation of IκB. Consequently, the freed NF-κB migrates into the nucleus to activate or repress expressions of responsive genes that mediate distinct biological functions. The activity of NF-κB is essential for T cell activation and proliferation, which requires plasma membrane recruitment of the IKK complex into lipid raft microdomain. This membrane recruitment process is critical in TCR-directed activation of NF-κB. The upstream serine kinase, PKCθ, plays an essential role in the process of the membrane translocation of the IKK complex. Although PKCθ does not directly interact with IKK, an intermediate protein complex comprising Carma1, Bcl10, and MALT1 was demonstrated to be critical to bridge IKK to PKCθ upon T cell activation. Depletion of Carma1 retards the membrane translocation of the IKK complex after TCR stimulation, whereas Bcl10 activates NF-κB through ubiquitination of IκκB.
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(20). These evidences provide critical insights on the roles of PKCθ, Carma1, and Bcl10 in TCR-directed activation of IKK and NF-κB. Lipid rafts are detergent-resistant cholesterol- and glycosphingolipid-enriched plasma membrane domains that can aggregate to form immune synapse upon TCR ligation (21–23). Enrichment of signaling molecules in this structure transmits activation signal to the downstream effector molecules. Although TNFα-induced activation of NF-κB can bypass PKCθ, this process requires membrane recruitment of the IKK complex and IKK-associated chaperone, Hsp90 (24).

In addition to the native inhibitors of NF-κB, IκBα, and the precursors of NF-κB1 and NF-κB2 (p105 and p100 respectively), several cellular factors including PP2Cβ, PP2A, A20, CYLD, hTid-1 (human Tid1), and Hsp70 have been reported to negatively modulate the activity of NF-κB through inhibition of IKK via distinct mechanisms (9, 25–31). Tid1 and Hsp70 form a molecular chaperone complex (32–34) in which Hsp70 is up-regulated during hyperthermia. Although in primary cells, the level of Hsp70 is low or even undetectable in the absence of stress, Hsp70 is abundantly expressed in most cancer cells, and this deregulated expression or activity is thought to play a prominent role in the process of oncogenesis (35, 36). Intriguingly, NF-κB is constantly activated in many types of cancer (37–41). Co-existence of the abundance of Hsp70 and the constitutive activity of NF-κB in cancer cells obviously contradicts the role of Hsp70 as a repressor to the IKK complex. To achieve a sustainable repression of NF-κB, an excessive amount of Hsp70 appeared to be needed (9). In fact, in most cancer cells the level of Hsp70 is already very high; heat shock does not further increase Hsp70 to a significantly higher level. Yet, the repression of NF-κB in heat-stressed cancer cells still occurs. Furthermore, we reproducibly observed that the repression of NF-κB took place immediately upon heat stress in T cells before de novo synthesis of Hsp70. These data imply that other events in addition to Hsp70 may play a role in the suppression of NF-κB during the physiological response to hyperthermia. In this study we explored the molecular mechanism of hyperthermia-associated repression of NF-κB in T cells. We found that heat stress caused rapid aggregation of Carma1 in lipid rafts, leading to subsequent membrane targeting of Bcl10 and PKCθ that in turn recruit downstream IKK and NF-κB signalosomes to the rafts. Sequestration of the IKK and NF-κB complexes in the lipid rafts and segregation of Hsp90 from the IKK complex lead to the inhibition of IKK, resulting in stabilization of NF-κB-IκBα complex in lipid rafts and a functional deficiency of NF-κB in T cells under hyperthermia.

EXPERIMENTAL PROCEDURES

Cell Lines, Antibodies, and Reagents—Human T cell lines including MT1, MT2, MT4, TL-Om1, SupT1, Jurkat, Jy1, and P116 were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum plus antibiotics at 37 °C, 5% CO2; the T cell lines OS-P2, FC36 were cultured in the same conditions as above with supplementation of natural IL-2 (10%). SupT1, Jurkat, E6-1, P116, and Jy1 cell lines were obtained from the ATCC (Manassas, VA), MT1, MT4, and TL-Om1 were kindly provided by Drs. Atsushi Koito and Takeo Oh sugi (Center for AIDS Research and Institute of Resource Development and Analysis, Kumamoto University). The Carma1-deficient Jurkat variant cell line, JPM50.6, was kindly provided by Dr. Xin Lin (M.D. Anderson Cancer Center, Houston, TX). The following reagent was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health: FC36.22 from Drs. Fiorenza Cocchi, Anthony DeVico, Alfredo Garzino-Demo, Suresh Arya, Robert Gallo, and Paolo Lusso (50); OS-P2/HTLV-I from Dr. Michael Lairmore (51); MT-2 from Dr. Douglas Richman. Peripheral blood lymphocytes were isolated from buffy coat of healthy blood donor using Ficoll-Hypaque method (GE Healthcare) following the manufacturer’s recommended protocol. The freshly isolated peripheral blood lymphocytes were stimulated with phytohemagglutinin (1 μg/ml) for 16 h, and then the cells were washed with RPMI1640 serum-free medium and cultured in the complete RPMI160 media supplemented with 10% normal IL-2 (ZeptoMetrix, Buffalo, NY) for an additional 7 days. After IL-2 stimulation, peripheral blood lymphocytes were cultured in the IL-2 free media for 2 days before heat shock procedure. Non-lymphoid cell lines HT1080 and human fibroblast cell line were obtained from the ATCC, and the mesothelial cell line was established by retroviral transduction of primary mesothelial cells with SV40 large T antigen.

Antibodies reacting to IκBα, IκBβ, p65, p50, p52, RelB, Bcl10, Hsp70, Hsc70, and Hsp90 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-IKKα, IKKβ, IKKγ, and serine-phosphorylated IκBα were purchased from IMGENEX (San Diego, CA). Anti-Carma1 and anti-phosphorylated IKKα/β were obtained from Cell Signaling Technology (Danvers, MA). Antibodies to PLCγ1 and ZAP70 were obtained from BD Transduction Laboratories, and LAT was from Upstate Biotechnology (Charlottesville, VA). Anti-β-actin antibody, horseradish peroxidase-conjugated cholera toxin β subunit, methyl-β-cyclodextrin (MβCD), protease and phosphatase inhibitor cocktails, and C6-ceramide were obtained from Sigma. The proteasome inhibitor MG-132 was from Calbiochem.

Mammalian Expression Plasmids—The expression plasmids for GST-tagged IκBα, IκBβ, p65 RelA, IKKα, IKKβ, and IKKγ were described previously (31). GST-tagged p50 was also constructed in the pBCAG vector. To generate the wild type of PKCθ, the full-length of PKCθ was amplified with PCR using Pfu DNA polymerase (Stratagene, La Jolla, CA), and the DNA template was from cDNAs prepared from human lymph node. The PCR fragment of PKCθ was digested with HindIII and BamHI and subsequently inserted into the expression vector pCEF with a C-terminal hemagglutinin tag and verified with DNA sequencing analysis. pRK6/Myc-Carma1 construct was provided by Dr. Xin Lin (M.D. Anderson Cancer Center).

Lentivirus Vector and Transduction—The full-length fragments of CD3ζ, IKKα, and IKKβ in the mammalian expression vectors were described previously (31, 52). The EcoRI/HindIII fragment of CD3ζ was fused with the HindIII/NotI fragments of either IKKα or IKKβ followed by ligation with lentivirus vector pLCMV6 that was pre-cut with EcoRI/NotI. The resulting recombinant lentivirus vectors comprised CD3-IKKα or CD3-IKKβ chimera driven by human cytomegalovirus promoter. A full-length of CD3ζ (EcoRI/NotI fragment) was inserted into the same lentivirus vector. The expressions of CD3ζ and the
CD3-IKK fusion genes were verified by transient transfection in HEK293 cells, and the activities of CD3-IKK were determined by the NF-κB luciferase reporter assay. To construct a known constitutively active IκB kinase, a HindIII-XbaI fragment containing the full-length of IκBβ(S177E/S181E) (constitutively kinase-active) in the vector pCMV2-IκBβ(S177E/S181E) (Addgene Inc., Cambridge, MA) was subcloned to the lentivirus vector with the GFP tag at the N terminus to generate GFP-IκBβ(S177E/S181E). This construct was verified with NF-κB reporter assay, fluorescence imaging, and immunoblot. The lentivirus production and transduction in T cell lines were followed with the procedure described previously (31), and ~10 multiplicity of infection was used for transduction of T cells with high efficiency. The full-length EcoRI/BamHI fragment of LAT derived from pCEF/LAT-FLAG (53) was fused with the enhanced GFP fragment (BamHI/XbaI) followed by insertion in the lentivirus vector that was pre-cut with EcoRI/XbaI. Transduction of MT2 cells was performed by the lentivirus carrying the LAT-GFP fusion gene. Expression of LAT-GFP in stably transduced MT2 cells was verified by visualization with fluorescence imaging and Western blot analysis using anti-GFP or anti-LAT antibody.

**Protein Interactions by GST Pulldown**—To examine the interaction of PKCθ with NF-κB signaling components in vivo, the PKCθ expression plasmid was co-transfected with GST-tagged IκBα and IκBβ and the subunits of NF-κB (p65 RelA and p50), IKKα, IKKβ, or IKKγ into HEK cells using SuperFect transfection reagent (Qiagen, Alencia, CA). 24 h post-transfection the cells were lysed in the Buffer A containing 1% Triton X-100, 40 mM Tris·Cl, pH 7.5, 150 mM NaCl, 2 mM MgCl₂, 0.5 mM dithiothreitol, and protease inhibitor mixture at 4 °C for 30 min. Glutathione-Sepharose beads were added into the soluble supernatants, and incubation was at room temperature for 2 h. The beads were then washed 3 times with the lysis buffer and subjected for SDS-PAGE plus Western blot analysis using anti-hemagglutinin (HA) antibody to detect HA-tagged PKCθ.

**Heat Shock Procedure and Western Blot Analysis**—The cells were left untreated or heat-shocked at 43 °C–45 °C/30 min followed by culturing at normal culture conditions (37 °C/5% CO₂) for the indicated time points. Cells were collected and lysed in the lysis buffer B containing 40 mM Tris·Cl, pH 7.6, 1% Triton X-100, 150 mM NaCl plus protease and phosphatase inhibitor cocktails at 4 °C for 30 min. The 1% Triton X-100-insoluble pellets were dissolved in the buffer C containing 1% SDS and 40 mM Tris·Cl, pH 8.0. Equal amounts of cellular proteins were analyzed by SDS-PAGE electrophoresis followed by immunoblot. Anti-β-actin blot was used for the protein loading control.

**Lipid Raft Fractionation by OptiPrep Density Gradient Ultracentrifugation**—Cells (4 × 10^7) were lysed in 2 ml of extraction buffer (20 mM Tris·Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 plus protease inhibitor mixture). Lysates were sheared by 20 passages through a 22-gauge needle, incubated for 20 min on ice before mixing with the OptiPrep density gradient medium (Iodixanol solution, final concentration, 40% vol/vol; AXIS-SHIELD PoC AS, Oslo, Norway), and placed at the bottom of a 12-ml tube. By overlaying 4 ml of 30% and 4 ml of 5% of OptiPrep medium, a discontinuous OptiPrep gradient was formed. Ultracentrifugation was performed at 100,000 × g for 4 h at 4 °C in SW41 rotor. 1 ml of each fraction from top to bottom was collected and subjected to Western blot analysis. Depletion of plasma membrane cholesterol by MβCD in Jurkat T cells was performed by pretreatment of the cells (4 × 10^7 cells each sample) with 10 mM MβCD for 45 min at 37 °C in Hanks’ balanced salt solution. After this step the cells were left unheated or heat-shocked. 30 min at the normal culture conditions after heat stress, cells were subjected to density gradient ultracentrifugation for lipid raft fractionation analysis. The pretreatment of Jurkat T cells was carried out by adding C6-ceramide (10 µM) into 4 × 10^7 cells/5 ml of medium for 30 min at 37 °C followed by heat shock procedure.

**Fluorescence Imaging**—The cells were cultured in a polylsine-treated dish at 37 °C, 5% CO₂ for about 20 min. The attached cells were washed with PBS and then fixed in iced methanolacetone (1:1) for 10 min at room temperature. Then the fixed cells were blocked with the horse serum-blocking solution and incubated with primary antibodies (1:100 in PBS) at 37 °C for 60 min. The cells were washed twice in PBS and then incubated for 30 min at room temperature with goat anti-mouse IgG-fluorescein isothiocyanate diluted 1:500 in PBS and 1 µg/ml Hoechst 33342. The cells were washed 3 times with PBS. Microscopy was performed using Leica TCS SP2 AOBS confocal microscope.

**Electrophoretic Mobility Shift Assay**—Nuclear extracts were prepared from MT2, Jurkat, and JPM50.6 cells before and after the heat shock procedure and with or without TNFα stimulation (10 ng/ml for 10 min at 37 °C) using NE-PER nuclear and cytoplasmic extraction reagents (Pierce). The sequence of the oligonucleotide corresponding to the κB element from IL-2 Rα gene was 5′-gatccGCGACGGGAAATCCTCCCTCTC-3′. The underlined sequence is the κB cis element. The oligonucleotide was 5′-end-labeled with biotin (Integrated DNA Technologies, Coralville, IA) and annealed to its complementary strand. The NF-κB binding activity to the κB element was examined by electrophoretic mobility shift assay (EMSA) using a LightShift chemiluminescent EMSA kit (Pierce). In brief, 5 µg of the nuclear extracts were preincubated in a 20 µl of total reaction volume containing 100 mM Tris, pH 7.5, 500 mM KC1, 10 mM dithiothreitol, 200 mM EDTA, pH 8.0, 50% glycerol, and 1 µg of polyoxyethylene-succinic-deoxyctydilic acid and 2 µl of biotin-labeled double-stranded probe (20 fmol) for 20 min at room temperature. Then the reactions were mixed with 5 µl of 5× loading buffer and run on a 6% nondenaturing polyacrylamide gel in 0.5× TBE (1× Tris-buffered EDTA: 89 mM Tris borate, 2 mM EDTA, pH 8.3) for 90 min at 100 V on ice. The binding reactions in the gel were transferred to Nylon membrane (Amersham Biosciences) at 380 mA for 1 h in 0.5× TBE on ice. The membrane was cross-linked at 100 ml/cm² for 2 min using UV-light cross-linker. Then the biotin-labeled DNA was detected by streptavidin-horseradish peroxidase and chemiluminescence.

**RESULTS**

**Both NF-κB and IκBα Shift from the Cytoplasm into Detergent-resistant Cellular Structure upon Heat Stress**—To investigate the role of the stress-induced Hsp70 in hyperthermia, we screened eight T cell lines to identify a model cell system that expresses a low or non-detectable level of Hsp70 at non-stress condition. Such a cell model would ideally produce an abun-
We next determined whether the shift of IκBα and NF-κB to the DR structure occurs in the early phase of hyperthermia response. As shown in Fig. 1D, regardless of pretreatment of Jurkat T cells with MG-132, large amounts of IκBα and NF-κB subunits, including p50 and p65, shifted from the soluble fractions into the DR structure as early as 20 min post-HS, whereas the level of Hsp70 remained constant regardless of heat shock. In MT1 cells, IκBα and NF-κB were confined in the DR even when Hsp70 was produced to a high level (Fig. 1, B and C). These results implicated that Hsp70 had no direct effect on the translocation of IκBα and NF-κB subunits into the DR nor did it cause redistribution of the translocated proteins in the DR structure. To verify this notion, we treated MT1 cells with cadmium chloride, which efficiently induced robust synthesis of Hsp70 4 h post-treatment, yet both IκBα and NF-κB (p50 NF-κB1 and p65 RelA) showed no visible loss in the soluble protein extracts (Fig. 1E). These data clearly indicate that the process of hyperthermia, rather than Hsp70, causes the shift of IκBα and NF-κB from the cytoplasm to the DR structure.

NF-κB and IκBα Are Recruited to the Membrane-associated Lipid Raft Microdomain in T Cells under Hyperthermia—The lipid raft microdomain is the detergent-resistant cholesterol- and glycosphingolipid-enriched membrane (GM1) structure. To verify whether or not IκBα and NF-κB translocate into this structure in heat-stressed T cells, the cellular protein extracts prepared from unheated and heat-shocked MT1 and MT2 cells underwent lipid raft fractionation analysis. In Fig. 2A, the results showed that IκBα and the subunits of NF-κB, p65RelA, and p50 were distributed predominantly in soluble fractions in unheated MT1 cells, although a fraction of p50 preexisted in the lipid raft fractions. However, these molecules shifted into the lipid raft fractions (fractions 4–6) in heat-stressed MT1 cells corresponding to the fractions of GM1 ganglioside (lipid raft marker, detected using horseradish peroxidase-conjugated cholera toxin β subunit).

In MT2 cells heat stress induced a rapid shift of IκBα from soluble fractions into the lipid raft fractions (fractions 5 and 6) (Fig. 2B). Notably, a doublet of IκBα, with the upper band representing the serine-phosphorylated IκBα as resulted from the constitutive hyperactivity of IKK in MT2 cells, was seen in the soluble fractions before HS, whereas only non-phosphorylated IκBα was detected in the lipid raft fractions after heat stress, suggesting that the activity of IKK was impaired during hyperthermia (Fig. 2B). Furthermore, rapid translocation of the NF-κB subunits (p50 and p65) from the soluble fractions into lipid raft was also observed in the heat-stressed MT2 cells (Fig. 2B), corresponding to the lipid raft biomarkers LAT (fractions 4 and 5) and GM1 fractions (fractions 4–6). These results support the notion that the NF-κB complex is indeed translocated to the membrane lipid rafts during hyperthermia stress.

IκB Kinases Are Translocated into Lipid Rafts during Hyperthermia—To investigate the possibility that IKK may be also recruited to the plasma membrane during hyperthermia stress, we performed similar experiments to examine the shifts of IκB kinases from soluble protein supernatants to the DR structure. Similar to the membrane recruitment of the NF-κB-IκBα complex, all three subunits of the IKK complex, including IκKα, IκKβ, and IκKγ, translocated from the soluble superna-
tant fractions into the DR structure in MT1 and Jurkat T cells as early as 20 min after heat shock (Fig. 3, A and B, respectively). Similar to the NF-κB-IκB complex, the rapid shift of IKK into the lipid raft was unlikely caused by Hsp70 since de novo synthesis of Hsp70 required a minimum of 2 h after stress. Analysis of heat-stressed MT1 cells with density gradient ultracentrifugation showed that the subunits of IKK shifted from the soluble fractions into lipid raft fractions (fractions 4–6) (Fig. 3C). Hsp70 was not detected in unheated MT1 cells but was induced 4 h after heat shock, yet newly synthesized Hsp70 was largely localized in the soluble fractions (Fig. 3C). Hsp90, a cofactor for the IKK complex, was reported to co-translocate with IKK into the lipid rafts in TNF-α-stimulated cells (24). We found that Hsp90 constitutively localized in the
soluble fractions regardless of heat shock (Fig. 3C). The shift of IKKs into lipid rafts appeared to be specific since mitogen-activated protein kinases such as ERK1 and JNK1 remained in the soluble fractions before or after HS (Fig. 3C). In MT2 cells a portion of IKKs was present in the lipid rafts before heat stress, whereas all three subunits of IKK accumulated in the lipid rafts immediately after heat stress (Fig. 3D). Hsp70 was present in the soluble fractions, and a very small amount of Hsp70 was able to relocate to lipid rafts in heat-shocked MT2 cells, whereas Hsp90 remained in the soluble fractions upon heat shock (Fig. 3D). Furthermore, fluorescence imaging analysis showed that both IKKα and NF-κB1 p50 were distributed in the cytoplasm, and some of p50 was in the nucleus before heat shock (Fig. 4A). However, both fluorescence signals of IKKα and p50 accumulated in the plasma membrane after heat stress (Fig. 4A). Double-staining results demonstrated that IKKα (red) co-localized with LAT (green) in the lipid rafts to yield a yellow cast in the merged picture in heat-stressed T cells (Fig. 4B). Taken together, it can be concluded that the signalosomes of both IKK and NF-κB are readily translocated into the membrane-associated lipid raft microdomain in T cells in response to heat stress.

PKCθ Directs Lipid Raft Recruitments of the NF-κB-IκB Complex in Hyperthermia—We explored the underlying mechanism of the membrane recruitment of the IKK and NF-κB complexes during heat stress. In resting T cells PKCθ is predominantly localized in the cytoplasm. Upon TCR activation, PKCθ can rapidly translocate to the membrane detergent-insoluble region or lipid rafts, which is one of the key molecules that directs the membrane recruitment of the IKK complex upon T cell activation. To determine whether heat stress utilizes a mechanism similar to TCR-mediated membrane recruitment of the IKK complex in T cells, we examined the potential shift of PKCθ from the cytoplasm into the DR structure in the stress condition. As shown in Fig. 5A, PKCθ was depleted in the soluble protein extracts (panel 1) and was accumulated in the DR structure (panel 3) in response to heat shock regardless of the presence of MG-132. PKCθ was rapidly recruited to the lipid rafts in both MT1 and MT2 T cells upon heat stress (Fig. 5B). By examining the interactions of PKCθ with the components of the IKK and NF-κB signalosomes, we found that PKCθ predominantly co-precipitated with IκBα and much less so with IκBβ in co-transfected HEK cells (Fig. 5C), whereas p65 RelA, p50, and three subunits of IKKs did not co-precipitate with PKCθ under the same conditions (Fig. 5C). Indeed, IκBα shifted to the plasma membrane, whereas IκBβ stayed in the soluble fractions during hyperthermia (Fig. 5D), implicating that the membrane recruitment of the NF-κB-IκB complex is through direct interaction of IκBα with PKCθ.

Membrane Lipid Raft Recruitment of PKCθ During Hyperthermia Is Dependent on Carma1—The membrane recruitment of PKCθ
recruitment of PKC

In Jurkat T cells, IKK upon heat stress, we utilized the Jurkat T cell model and the analysis.

uble fractions 8 and 9 after heat shock, as shown by lipid raft fractionation

ies showed that the Carma1, Bcl10, and MALT1 complex, variant cell line, JPM50.6, the lipid raft translocations of PKC

6

and

IKK (IKK

B)

in the lipid rafts during hyperthermia stress. E, accumulation of Carma1 and Bcl10 in lipid rafts in heat-stressed Jurkat T cells, whereas Bcl10 remained in the soluble cytoplasmic fractions in the Carma1-deficient Jurkat variant, JPM50.6 cells, after HS.

typically occurs in T cell activation. The finding that PKC\(\theta\) is targeted to the membrane in heat stress raised a possibility that both hyperthermia and T cell activation share a similar mechanism for the membrane targeting of PKC\(\theta\). The previous studies showed that Carma1, Bcl10, and MALT1 complex, which consists of Carma1, Bcl10, and MALT, serves as scaffold proteins to connect IKK to PKC\(\theta\) (42). Carma1 (CARD11) was shown to be critical for activation of NF-\(\kappa\)B in TCR signaling (43), and loss of Carma1 in Jurkat T cells impaired membrane recruitment of PKC\(\theta\), IKK\(B\), and Bcl10 upon T cell activation (19, 44), indicating that Carma1 plays an essential role in recruiting IKK to the membrane through PKC\(\theta\). To examine the role of Carma1 in the lipid raft recruitment of PKC\(\theta\) and IKK upon heat stress, we utilized the Jurkat T cell model and the Carma1-deficient Jurkat variant, JPM50.6 (19). In Jurkat T cells, hyperthermia caused a drastic shift of PKC\(\theta\) and the downstream NF-\(\kappa\)B signaling molecules, including three subunits of IKK (IKK\(\alpha\), IKK\(B\), and IKK\(\gamma\)), the NF-\(\kappa\)B heterodimer (p65/50), and I\(\kappa\)B\(\alpha\), to the lipid rafts (Fig. 6A, fraction 5, corresponding to the fraction containing the lipid raft protein marker, LAT). However, in JPM50.6 cells, PKC\(\theta\) was retained in the soluble fractions after heat shock (Fig. 6B). Consequently, the membrane translocation of the subunits of IKKs, NF-\(\kappa\)B, and I\(\kappa\)B\(\alpha\) was completely retarded in the Carma1-deficient T cells (Fig. 6B). These results strongly indicate that Carma1 plays an essential role for the lipid raft translocation of PKC\(\theta\) in T cells during hyperthermia stress.

We next applied the chemical inhibitor of cholesterol, M\(\beta\)CD, which can effectively deplete cholesterol on the plasma membrane, consequently impairing the membrane lipid raft recruitment of signaling molecules such as PKC\(\theta\). Indeed, pretreatment of T cells with M\(\beta\)CD completely blocked the shift of PKC\(\theta\) from the soluble fractions to the lipid raft fractions. Instead, PKC\(\theta\) accumulated in the non-lipid raft fractions after heat shock (Fig. 6C). Accordingly, IKK\(\alpha\), IKK\(B\), IKK\(\gamma\), p50, and I\(\kappa\)B\(\alpha\) were concentrated in the non-lipid raft fractions (Fig. 6C). Next, we utilized a PKC\(\theta\) inhibitor, C6-ceramide (45). Jurkat cells were pretreated with C6-ceramide (10 \(\mu M\)) for 30 min at 37 °C before heat shock, and the results showed that C6-ceramide impaired accumulation of PKC\(\theta\), IKK\(\alpha\), and IKK\(B\) as well as the NF-\(\kappa\)B-I\(\kappa\)B\(\alpha\) complex into the lipid rafts (fraction 5) in response to heat stress (Fig. 6D). These results are in strong support of the crucial role of PKC\(\theta\), directed by Carma1, in the lipid raft recruitment of the IKK and NF-\(\kappa\)B-I\(\kappa\)B\(\alpha\) complexes under hyperthermia. Furthermore, we determined whether heat stress affects the behavior of Carma1. It has been shown that a portion of Carma1 was found to be constantly associated with lipid rafts (46). We showed that Carma1 was distributed predominantly in soluble fractions in Jurkat T cells before heat stress but was rapidly aggregated in the lipid rafts after heat shock (Fig. 6E). In accordance with the aggregation of Carma1, Bcl10 was shifted to the rafts in Jurkat cells (Fig. 6E). In contrast, Bcl10 remained in the soluble fractions regardless of heat stress in the Carma1-deficient JPM50.6 cells, indicating the accumulation of Carma1 in the rafts is essential for the membrane translocation of Bcl10. Thus, Carma1 is the key player that recruits both PKC\(\theta\) and Bcl10 in promoting membrane translocation of downstream IKK and NF-\(\kappa\)B complexes in T cells under hyperthermia.

Membrane Translocation of PKC\(\theta\) during Heat Stress Is Independent of ZAP70 and PLC\(\gamma1\)—In the proximal signaling events of TCR activation, activation of Lck, the first line of the protein tyrosine kinases, initiates signaling cascade by sequentially activating downstream kinases including ZAP70 and PI3 kinase, which in turn activate PLC\(\gamma1\). LAT-dependent membrane recruitment of PLC\(\gamma1\) induces its lipase activity to generate secondary lipid messengers including diacylglycerol, which activates PKCs. To determine whether the membrane translocation of PKC\(\theta\) during hyperthermia is dependent of the proximal factors, we first evaluated a possible membrane accumulation of signaling molecules that is essential in TCR signaling using primary peripheral blood lymphocytes. Heat shock indeed induced a dominant shift of PKC\(\theta\) to the lipid rafts, whereas no apparent migration of upstream kinases ZAP70 and PLC\(\gamma1\) to the lipid rafts was observed in heat-stressed primary lymphocytes (Fig. 7A). Accordingly, the subunits of IKK and NF-\(\kappa\)B1 (p50) as well as the precursor of NF-\(\kappa\)B1, p105, were
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FIGURE 7. The membrane translocation of PKCθ is independent of ZAP70 and PLCγ1 during hyperthermia. A, lipid raft fractionation assay showed the movement of PKCθ, IKKs, and p50/p105, but not ZAP70, PLCγ1, Hsp90 or ERK1, after heat stress in primary lymphocytes. B, in the ZAP70-deficient Jurkat variant, P116, heat stress induced migration of PKCθ and IKKs to the lipid raft fractions, corresponding to the lipid raft-bound LAT. C, lipid raft fractionation of the PLCγ1-deficient Jurkat variant, Jγ1, showed that the membrane translocation of PKCθ and IKKs still occurred in response to heat stress.

accumulated in the lipid rafts although these movements were less potent than in established T cell lines (Fig. 7A). Similar to the finding in T cell lines, Hsp90 and ERK1 remained in the soluble fractions regardless of heat stress (Fig. 7A). These results suggest that the membrane translocation of PKCθ is independent of its proximal factors such as ZAP70 and PLCγ1.

To examine this notion further, we utilized two Jurkat variant lines, P116 (ZAP70-deficient) and Jγ1 (PLCγ1-deficient). As shown in Fig. 7B, the lipid raft fractionation assay demonstrated that the accumulation of PKCθ in the lipid rafts after heat stress still occurred in P116 cells, and accordingly, the membrane recruitment of the IKK complex was also seen. These results suggest that although ZAP70 is essential for TCR-directed activation, it is dispensable for hyperthermia-induced membrane translocation of PKCθ. Similarly, in PLCγ1-deficient Jurkat variant line, Jγ1, heat stress induced robust membrane lipid raft translocation of PKCθ as well as the downstream IκB kinases (Fig. 7C). Furthermore, we found that the essential T cell adaptor protein, LAT, was not tyrosine-phosphorylated in response to heat stress (data not shown). These results strongly indicate that unlike the proximal events of TCR activation, neither ZAP70 nor PLCγ1 plays a crucial role for the membrane targeting of PKCθ in the stress condition. Instead, heat stress targets primarily at Carm1 in sequestrating downstream IKK and NF-κB complexes in lipid rafts.

Hyperthermia Represses the Activity of IKK in Lipid Rafts in T Cells—TCR stimulation induces activation of NF-κB, whereas heat stress leads to the inhibition of NF-κB in numbers of solid tumor cell lines. The rapid transition of serine-phosphorylated IκBα to the non-phosphorylated form was seen in heat-stressed MT2 T cells (Fig. 3B), indicating that the activity of IKK is compromised. It was noted that the catalytic activity of IKK in the lipid rafts could not be assessed by the in vitro kinase assay because the purification of these kinases from this structure would impair their activities, which certainly affects interpretation of the activation status of IKK in living cells. Because the phosphorylation of IκBα on its N-terminal serine residues is exclusively mediated by the catalytic subunits of IKK, utilization of the antibody that reacts specifically on the serine-phosphorylated IκBα is a strong indication for the activity of IKK in the lipid rafts in T cells.

We first examined the serine phosphorylation status of IκBα in response to TNFα. In MT1 cells, a very small fraction of total IκBα protein was serine-phosphorylated, implicating that IKK was marginally activated (Fig. 8A, upper panel, lane 1). The phosphorylation of IκBα was enhanced by TNFα treatment (Fig. 8A, upper panel, lanes 2 and 3). Surprisingly, the phosphorylation of IκBα was completely blocked as early as 30 min post-HS, far before Hsp70 was produced, and IκBα was no longer phosphorylated in heat-stressed MT1 cells upon TNFα stimulation (Fig. 8A, upper panel, lanes 4 – 6). Moreover, induction of Hsp70 did not lead to re-phosphorylation of IκBα in response to TNFα (Fig. 8A, upper panel, lanes 7 – 9), suggesting that de novo synthesis of Hsp70 neither suppresses nor rejuvenates the activity of IKK. Furthermore, heat stress caused reduction of IκBα in the detergent-soluble supernatants, whereas IκBα rapidly shifted into the DR structure or lipid rafts (Fig. 8A, lower panel). Yet the IκBα protein in lipid rafts was unable to be phosphorylated by TNFα treatment in heat-stressed cells (Fig. 8A, lower panel, lanes 4 – 9), which suggests that the activity of IKK is prohibited under hyperthermia condition. In MT1 cells, ERK1/2 were constitutively activated, as revealed by their specific serine phosphorylation, and heat stress had no effect on the intensity of the phosphorylation of ERK1/2 (Fig. 8B). This implies that hyperthermia causes specific repression on IKK but has no inhibitory effect on ERK1/2.

In MT2 cells the IκBα protein was low due to its rapid turnover but was stabilized in the cells pretreated with the proteasome inhibitor, MG-132. A vast majority of IκBα was serine-phosphorylated in MT2 cells before heat shock, and phorbol 12-myristate 13-acetate/ionomycin (P/I) treatment did not further increase the intensity of the phosphorylated IκBα (Fig. 8C, lanes 1 – 3). Heat stress caused the shift of IκBα from the cytoplasm to the lipid rafts as early as 30 min post-HS, and the IκBα protein in both soluble supernatants and lipid rafts was in a non-phosphorylated form even with P/I stimulation (Fig. 8C, lanes 4 – 6). We also found that in MT2 cells, IKKα was present in the lipid rafts in the phosphorylated forms before heat shock, indicating these kinases were constitutively activated (Fig. 8D). Heat stress induced rapid accumulation of IKKα in the lipid rafts (Fig. 8D, lower panel) and inhibited the phosphorylation of IKKα in this structure (Fig. 8D, upper panel). Thus, it is apparent that the process of hyperthermia, not the synthesis of Hsp70, executes a complete blockade of the serine phosphorylation of IκBα by rapidly inhibiting the activity of IKK.
Hyperthermia Response in T Lymphocytes

To further examine the notion that the activity of IKK is prohibited in the rafts during hyperthermia, we constructed membrane-targeted chimeras of IKKα and IKKβ that were fused with CD3ζ to generate CD3-IKKα and CD3-IKKβ, respectively. The fusion of IKK with CD3ζ targets IKK to the plasma membrane in the absence of TCR activation or hyperthermia, as it is agreed that a portion of TCR-CD3 clusters localizes in the lipid rafts in quiescent T cells (47). In addition, these chimeras acquired catalytic activities of IKKα and IKKβ as seen in transfected HEK cells without extracellular and intracellular stimuli (data not shown). In T cells that stably expressed CD3, CD3-IKKα, or CD3-IKKβ, heat stress resulted in instant suppression of IκBα phosphorylation in all three established cell lines (Fig. 8E). The heat-shocked cells were unable to respond to TNFα-mediated activation of IKK (Fig. 8A). Next, the κB DNA binding activity of the nuclear extracts before and after heat stress was examined with electrophoretic mobility shift assay. As shown in Fig. 8G, the basal activity of the κB binding of NF-κB in MT2 cells was high, and this activity was inhibited after heat stress. In T cells that exogenously expressed a constitutively kinase-active form of IKKβ (S177E/S181E), heat stress still potently suppressed the activity of NF-κB and diminished the phosphorylation of IκBα (Fig. 8, B and I), and both IKKβ(S177E/S181E) and IκBα were shifted into the detergent-resistant structure (Fig. 8I, lower panel). Furthermore, the basal activity of κB binding was almost undetectable in Jurkat T cells and was readily induced by TNFα before heat shock (Fig. 8I, lane 2), and heat stress resulted in the unresponsiveness of these cells to TNFα-induced NF-κB activity (Fig. 8I, lane 4). Remarkably, the Carma1-deficient, Jurkat variant cells (JPM50.6) responded to TNFα-mediated activation of IKK (Fig. 8A). Next, the κB DNA binding activity of the nuclear extracts before and after heat stress was examined with electrophoretic mobility shift assay. As shown in Fig. 8G, the basal activity of the κB binding of NF-κB in MT2 cells was high, and this activity was inhibited after heat stress. In T cells that exogenously expressed a constitutively kinase-active form of IKKβ (S177E/S181E), heat stress still potently suppressed the activity of NF-κB and diminished the phosphorylation of IκBα (Fig. 8, B and I), and both IKKβ(S177E/S181E) and IκBα were shifted into the detergent-resistant structure (Fig. 8I, lower panel). Furthermore, the basal activity of κB binding was almost undetectable in Jurkat T cells and was readily induced by TNFα before heat shock (Fig. 8I, lane 2), and heat stress resulted in the unresponsiveness of these cells to TNFα-induced NF-κB activity (Fig. 8I, lane 4). Remarkably, the Carma1-deficient, Jurkat variant cells (JPM50.6) responded to TNFα-mediated NF-κB activity regardless of heat stress (Fig. 8K, lanes 2 and 4). Together, these results indicate that the kinase activity of IKK was impaired under hyperthermia stress in T cells in Carma1-dependent manner, and such repression of IKK can occur in the lipid rafts.

Finally, we tested three non-lymphoid cell lines and found that heat stress did not cause the lipid raft translocation of IκB kinases (Fig. 9A). To determine whether expression of Carma1...
alone in non-lymphoid cells is sufficient to mediate heat stress-induced inhibition of IκB kinases, we expressed Carma1 into HT1080 cells. We found that expression of Carma1 in HT1080 cells did not lead to the lipid raft translocation of IKK, and these cells still responded to TNFα-mediated NF-κB activation (Fig. 9, B and C). These data indicated that Carma1 is a critical determinant for the induction of the lipid raft translocation of NF-κB signaling complexes in T cells upon heat stress, whereas Carma1 alone is not sufficient to mediate lipid raft translocation and inactivation of NF-κB in non-lymphoid cells.

DISCUSSION

In this study we illustrated a novel mechanism of hyperthermia-associated repression of NF-κB in T lymphocytes. Heat stress causes rapid membrane lipid raft recruitment and sequestration of the IKK and NF-κB-IκB complexes. In addition, the segregation of the IKK complex from Hsp90 leads to the prohibition of the activity of IKK, resulting in blockade of the serine phosphorylation of IκBα catalyzed by IKK. These coordinated actions suppress the activity of NF-κB for at least 6 h after heat shock. Heat stress renders T cells resistant to TNFα-induced activation of IKK (see the hypothetical model in Fig. 10).

Our data showed that Hsp70 does not play a major role in the repression of NF-κB in the early phase of hyperthermia response in T cells. Although Hsp70 was reported to interact with IKKγ, how this interaction leads to repression of the catalytic subunits of IKKs, IKKα or IKKβ, remains unsolved. To explore the molecular mechanism of hyperthermia response in T cells, we utilized several T cell model systems that provide dynamic views for the fate of NF-κB signaling molecules during heat stress. First, Hsp70 is not detected in MT1 cells within 2 h post-HS, so this leaves a 2-h time window for assessing an immediate response to heat stress by excluding potential effects of Hsp70. Second, IKK is constitutively activated, and IκBα is serine-phosphorylated in MT2 cells so the repression of IKK can be determined in the earliest stage of hyperthermia response. Third, Jurkat T cell model and its variant cell lines are utilized to determine the key signaling events during hyperthermia. And last, the use of primary lymphocytes provides a physiological relevance of hyperthermia response. We showed that rapid translocation of IKK and the NF-κB-IκB complexes to the rafts occurs as early as 20 min, and sequestration of these molecules in this compartment persists for at least 6 h post-heat stress. Consequently, the serine phosphorylation of IκBα in response to TNFα stimulation is abolished regardless of the presence of Hsp70. Thus, in contrast to the previous reports for the role of Hsp70 in hyperthermia-associated repression of NF-κB in adherent cells, our data indicate that the repression of NF-κB is not related to Hsp70 in T cells. Rather, hyperthermia induces rapid membrane lipid raft recruitment and sequestration of the IKK and NF-κB signalosomes in T cells.

We identified PKCθ and Carma1 as primary factors directing the membrane recruitment of the IKK and NF-κB complexes during hyperthermia. By analyzing the signaling molecules recruited to the lipid rafts in T cell activation and hyperthermia, both events depend on Carma1 in recruiting the IKK complex to the plasma membrane. The membrane recruitment of PKCθ, Bcl10, and the IKK and NF-κB signalosomes is completely defective in the Carma1-deficient T cells upon heat stress. Moreover, the cholesterol inhibitor, MβCD, effectively prevents the lipid raft translocation of PKCθ from the cytoplasm. Furthermore, C6-ceramide impaired the membrane translocation of PKCθ, IKK, and NF-κB complexes in response to heat stress. Our results showed that heat stress caused aggregation of Carma1 in the lipid rafts, which sequentially recruits PKCθ, Bcl10, and the IKK and NF-κB complexes but not the cofactor Hsp90 to the rafts. These data, therefore, validate the mechanism of Carma1-dependent, PKCθ-directed, membrane lipid raft recruitment of the IKK complex upon heat stress in T cells. Although the hyperthermia response in B lymphocytes has not been explored in this study, studies on B cell receptor signaling showed that activation of B cells can recruit IKK to lipid rafts through PKCβ (48), suggesting that a similar scenario to T cells may occur in B cells. Both ZAP70 and PLC-γ1 are required for activation of PKCθ in TCR activation (49) but are dispensable for the membrane recruitment of PKCθ and IKK under the
stress condition. Indeed, our data demonstrated that heat stress does not engage TCR; instead this stress process primarily targets at Carma1 in T cells, leading to the membrane recruitment of PKCθ and sequestration of the IKK complex. Because Hsp90 is an essential cofactor for the interactions, Hsp90 remains in the cytoplasm, segregating itself from the resting stage or the melting pot for enrichment of signaling ner. Lipid rafts, therefore, can serve as the physical shield in the downstream signaling molecules, and this mode of action pre-

Hsp90 from the IKK complex. Lipid rafts can serve as the phys-

lipid rafts.

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