Co-localization of CD3 and prion protein in Jurkat lymphocytes after hypothermal stimulation

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Abstract While long-term effects of temperature treatment in respect of, e.g., gene-expression and cellular function have already been studied in some detail, nothing is known on the physiological responses of lymphocytes during short-term hypothermal shifts. In this report, we characterized the effects of such a stimulation using the human lymphocyte cell line Jurkat E6.1 and present evidence that warming from 4 to 37 °C for only 2 min is sufficient to cause co-localization of CD3, prion protein and the lipid-raft ganglioside GM1 paralleling lymphocyte activation as observed by Ca^{2+} mobilization and mitogen-activated protein kinase-phosphorylation.

Keywords: Lymphocyte; T-cell receptor; Prion protein; Hypothermal stimulation; Lipid raft; Fluorescence microscopy

1. Introduction

The activation of T-lymphocytes occurs primarily through the well-characterized mechanism of antigen-mediated T-cell receptor (TCR) clustering [1]. However, secondary priming routes like the stimulation through cytokines or unspecific stress-signals (e.g., temperature, oxidative stress and irradiation) are gaining increasing interest [2–4]. The earliest detectable biochemical events after lymphocyte activation include rapid tyrosine phosphorylation of multiple intracellular proteins leading to the triggering of downstream signaling pathways [5]. These include, but are not limited to, hydrolysis of inositol-containing phospholipids, Ca^{2+} mobilization and activation of the mitogen-activated protein kinase (MAPK) pathways [6]. Immediate responses of lymphocytes following specific stimulation like the appearance of an immunological synapse or the secretion of lymphokines have been studied extensively in terms of the above-mentioned signal transduction cascades (for reviews see [7,8]). Yet, experiments using unspecific stimulation through temperature treatment have until now focused primarily on long-term effects and investigated the response of lymphocytes in respect of, e.g., proliferative outcome, lymphokine production or alterations in gene expression patterns [9,10]. For instance, the down-regulation of TCR-ζ chain expression in lymphocytes and the up-regulation of heat-shock protein 70 as well as prion protein (PrP) expression in human NT-2 cells have been reported [11–13].

The aim of our work was to study the early responses of lymphocytes following hypothermal stimulation. Such treatments have relevance in a variety of applications, ranging from the areas of transplantation and critical care medicine into the experimental field of designing cellular assay systems. While the physiological effects of the first are only beginning to be studied in more detail, the incubation of cells at low temperatures is a standard procedure in cell biology. We have selected the CD3ϵ-subunit of the TCR-complex as an exemplary protein necessary for ligand-specific lymphocyte activation and investigated its behavior during hypothermal treatment. In addition, the glycosylphosphatidylinositol (GPI)-anchored PrP was chosen because of its preferential localization in lipid-raft microdomains and due to its possible roles during T-lymphocyte activation [14–16] (for reviews see [17–19]). We have focused on early events following hypothermal stimulation in terms of reallocation of the selected proteins into aggregating membrane domains as well as cell-activation in respect of Ca^{2+} mobilization and MAPK/stress-activated protein kinase (SAPK)-phosphorylation.

2. Materials and methods

2.1. Cell culture, reagents and antibodies

The human T-cell lymphoma cell line Jurkat E6.1 was cultured in RPMI-medium containing 10% heat-inactivated fetal bovine serum (Summit Biotechnology™), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (all reagents GIBCO™, Invitrogen Cooperation). The enhanced chemiluminescence (ECL) detection kit and fluorescent labeled secondary antibodies were from Amersham Biosciences. Mouse monoclonal anti-PrP antibody (SAF-32) was purchased from Cayman Chemical, mouse monoclonal anti-CD3 antibody and Alexa Fluor 647-labeled anti-CD3 Fab-fragments were gifts from Dr. H. Stockinger (Institute of Immunology, University of Vienna, Austria). Calcium Green-1, Alexa Fluor 488-labeled transferrin and Alexa Fluor 647-labeled cholera toxin subunit B (CT-B) conjugate were from Molecular Probes Inc., methyl-β-cyclodextrin (MβCD), cytochalasin D (Cyt D) and all other antibodies were from SIGMA®.

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Abbreviations: TCR, T-cell receptor; GPI, glycosylphosphatidylinositol; PrP, prion protein; MAPK, mitogen-activated protein kinase; SAPK, stress-activated protein kinase; MβCD, methyl-β-cyclodextrin; CT-B, cholera toxin subunit B

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2.2. Hypothermal treatment of cells, immunofluorescence staining and life cell imaging

Cells growing in log-phase were adjusted to a concentration of 7×10^6 cells/ml in Opti-MEM® I medium (GIBCO™) and incubated on ice for 30 min. After warming to 37 °C for 2 min (except indicated otherwise), cells were fixed in 3.7% formaldehyde in PBS for 15 min at RT and spun onto poly-L-lysine-coated cover glasses (Cytospin, Thermo Shandon Inc.). For cell surface staining of the ganglioside GM1, cells were incubated with 1 μg/ml Alexa Fluor 647-labeled CT-B in PBS for 15 min at RT. After washing with PBS, cover glasses were sealed using HybriWell™ chambers (GRACE Bio-Labs Inc.). Cell surface transferrin receptor was stained with 100 μg/ml Alexa Fluor 488-labeled transferrin in 1% BSA/PBS for 30 min at 37 °C on cells depleted of endogenous transferrin by serum starvation for 45 min at 37 °C in HBSS. For cell surface staining of PrP and CD3, cells were blocked with 2% BSA/PBS for 20 min and incubated with the appropriate primary antibodies (anti-PrP 0.2 μg/ml; anti-CD3 5 μg/ml) for 30 min. After incubation with Cy3-labeled secondary antibodies (1:2000) for 30 min, samples were sealed as described above. For life cell imaging of intracellular free Ca^{2+}, cells were incubated with 10 μM Calcium Green-1 in normal growth medium for 15 min at 37 °C, followed by incubation with Alexa Fluor 647-labeled anti-CD3 Fab-fragments (5 μg/ml) for 30 min at 4 °C. After washing cells in ice-cold HBSS they were flushed into a perfusion chamber prewarmed to 37 °C. Ca^{2+} images were acquired every 2 s and cap formation was verified by CD3 cell surface distribution 2–3 min after start of experiments.

2.3. SDS–PAGE and Western blot analysis

Western blot analysis was performed as described earlier [20]. Equal amounts of protein were subjected to 10% SDS–polyacrylamide gel electrophoresis and transferred to PVDF membranes (Immobilon™-P; Millipore Corporation). After blocking in PBS containing 0.1% Tween 20 and 5% non-fat dry milk for 1 h, membranes were incubated with the appropriate primary antibodies overnight at 4 °C at the dilution suggested by the supplier. Following incubation with peroxidase conjugated secondary antibodies for 1 h, signal was developed by use of the ECL reagent (Amersham Biosciences).

2.4. Inhibition experiments

Raft structures were disturbed by treatment with 4 mM MβCD for 40 min at 37 °C prior to hypothermal stimulation. Viability of cells was tested by trypan blue exclusion staining. No significant cell death was detected after cholesterol extraction (data not shown). For disruption of the actin–cytoskeleton, cells were preincubated with 10 μM Cyt D for 20 min in Opti-MEM® I medium prior to hypothermal treatment.

2.5. Fluorescence microscopy and image processing

To analyze large populations of Jurkat E6.1 cells in these experiments, we utilized a self-developed fluorescence screening system (CytoScout®, Upper Austrian Research GmbH). The system is based on a conventional epi-fluorescence microscope (Axiovert 200, Zeiss) using wide field illumination and a motorized scanning stage (Märzhäuser GmbH). Scans of usually 1×1 mm allowed for representative analysis of our experiments and were facilitated by a special read-out mode (patent pending; for details see [21]). Image processing was based on a pattern matching algorithm programmed in C++ using the Matrox Imaging Library (Matrox Electronic Systems Ltd.). For analysis, a typical cap was selected as a template and parameters of the algorithm were adjusted such that a good agreement of identified caps with numbers obtained by visual inspection was achieved. Experiments were repeated at least three times (statistical analysis by Student's *t*-test) and results of representative experiments are shown in the figures.

3. Results

3.1. Clustering of GM1-rich microdomains is induced by hypothermal stimulation

We studied the distribution of the raft-enriched glycosphingolipid GM1 in Jurkat E6.1 cells during hypothermal treatment. GM1 is evenly distributed on the plasma membrane of untreated cells or cells incubated at 4 °C only, whereas a temperature shift from 4 to 37 °C leads to the formation of cap-like structures enriched in GM1 in approximately $30 \pm 2\%$ of cells ($P < 0.0001$ versus control $5.5 \pm 2.6\%$; Fig. 1A). Also lesser temperature differences were tested. Incubation at 12 °C for 30 min prior to 37 °C warming was still sufficient to induce membrane aggregation in 21% of the cells. When the temperature was changed from 20 or 28 to 37 °C, almost no cap-structures could be detected (9% and 10%, respectively). First reorganization of the plasma membrane can be observed already 1 min after temperature change (Fig. 1B). Cap-like structures are fully developed after 2 min and disappear when the 37 °C incubation period is prolonged.

3.2. Co-localization of CD3 and PrP with GM1

To further characterize these temperature-induced structures, we investigated the distribution of the TCR-component CD3ϵ as well as the GPI-linked membrane protein PrP on the membrane of stimulated lymphocytes. In control experiments, fluorescent labeled transferrin was used to study the distribution of the transferrin receptor, a non-raft molecule. Following incubation at 4 °C for 30 min and warming to 37 °C for 2 min, CD3 and PrP co-localized in fully developed GM1-rich cap-structures. In contrast, transferrin remained evenly distributed over the whole cell surface, providing evidence for a reorganization of raft microdomains (Fig. 2).

3.3. Activation of the MAPK-pathway by hypothermal stimulation

To investigate whether cap-formation is associated with the activation of signal transduction cascades, we tested the

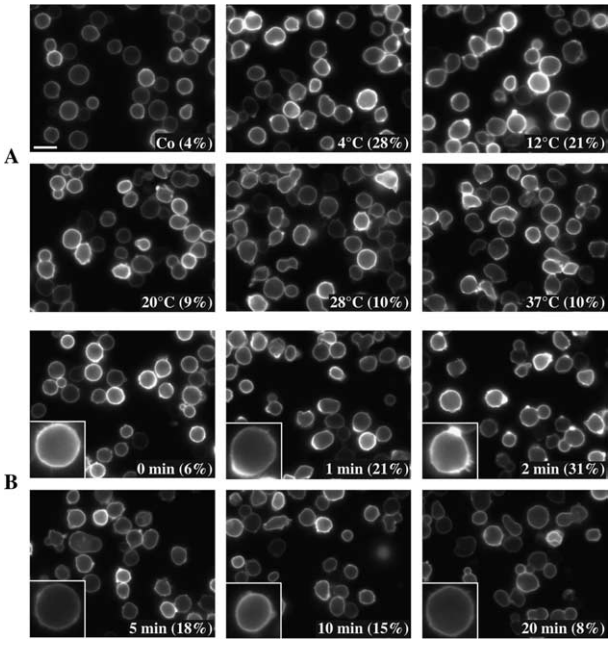

Fig. 1. Clustering of GM1-rich domains after hypothermal stimulation. (A) Effects of temperature shifts. Cells were incubated at the indicated temperatures for 30 min, warmed to 37 °C for 2 min, fixed and stained for cell surface GM1 with Alexa Fluor 647-labeled CT-B. Fluorescence images were analyzed for the number of caps in relation to the whole cell number (% in brackets). (B) Time course of cap formation. Cells were incubated at 4 °C for 30 min, warmed to 37 °C for the times indicated and analyzed as described above. Representative cells are magnified. Bar, 20 μm.

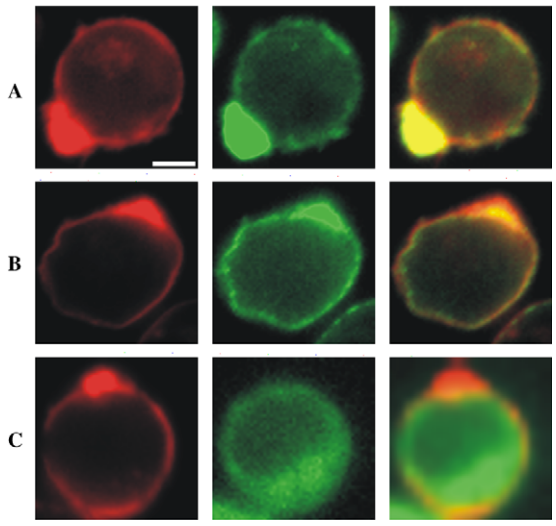

Fig. 2. Co-localization of CD3 and PrP with GM1-rich domains. Cells stimulated by hypothermal treatment were fixed and stained for (A) GM1 (red) and CD3 (green), (B) GM1 (red) and PrP (green) and (C) GM1 (red) and transferrin (green) as described in Section 2. Shown is the fluorescence image of a representative cell (left and middle column) as well as the merge of the two signals (right column). Bar, 5 μm.

phosphorylation of different members of the MAPK family: p44/42 MAPK, SAPK and p38 MAPK. Activation of MAPKs was determined by immunoblotting using antibodies specific for the phosphorylated forms of the respective enzymes. Total levels of endogenous p44/42 MAPK were used as a control for equal loading. Maximal p44/42 MAPK phosphorylation was obtained already after 1 min of 37 °C treatment following hypothermal stimulation, reaching basal levels again after 10 min (Fig. 3). In contrast, no phospho-SAPKs or phospho-p38 MAPKs were detected (data not shown). The time course of p44/42 MAPK activation mirrors that of cap formation (compare Figs. 1B and 3).

3.4. Inhibition of capping by treatment with MβCD or Cyt D

To determine the necessity of intact lipid-raft structures during hypothermal treatment, Jurkat E6.1 cells were depleted of cellular cholesterol by incubation with 4 mM MβCD [22]. Subsequently, cells were stimulated as described above and stained for GM1. Formation of cap-structures was abolished completely in Jurkat E6.1 cells pretreated with MβCD (Fig. 4A) indicating that intact lipid rafts are essential for reallocation of the selected proteins. In contrast, p44/42 MAPK phosphorylation was not impaired as shown in Fig. 4C. We also investigated whether an intact actin cytoskeleton is required for the formation of cap-like structures.

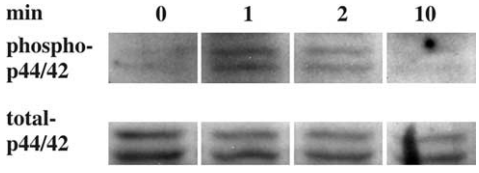

Fig. 3. Activation of the MAPK-pathway by hypothermal treatment. Cells were treated as described in Fig. 1B and cell lysates were immunoblotted with anti-phospho-p44/42 (upper panel) and anti-p44/42 antibodies (lower panel) as described in Section 2.

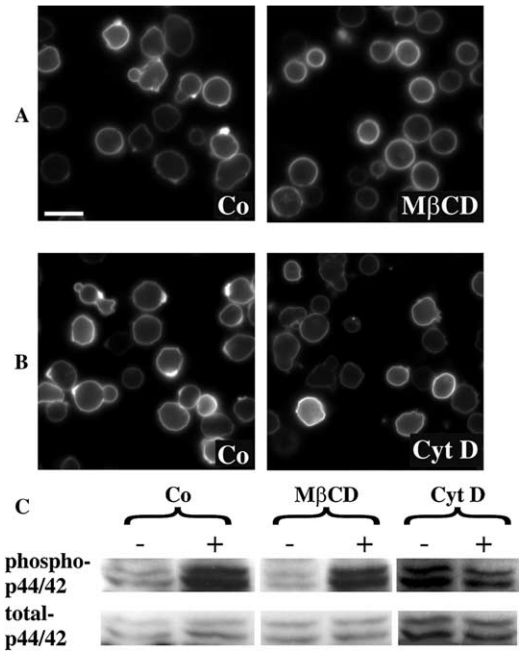

Fig. 4. Influence of cholesterol and actin on temperature-induced cap-formation. (A) Depletion of cellular cholesterol with MβCD. Cells were maintained without (Co), or with 4 mM MβCD at 37 °C for 40 min prior to hypothermal treatment and stained for GM1 as described in Section 2. (B) Disruption of the actin cytoskeleton by Cyt D. Cells were incubated without (Co), or with 10 μM Cyt D for 20 min at 37 °C and stained for GM1. (C) Cells were incubated without (Co), or pretreated either with MβCD or Cyt D as described above, washed, incubated at 4 °C for 30 min (−) or subsequently warmed to 37 °C for 2 min (+). Cell lysates were prepared and analyzed by immunoblotting with the indicated antibodies. Bar, 20 μm.

Incubation of Jurkat E6.1 cells with 10 μM Cyt D preceding hypothermal stimulation prevented the capping process (Fig. 4B). A possible influence of the cytoskeleton on the hypothermal activation could not be studied because Cyt D alone caused high levels of phosphorylated p44/42 MAPK (Fig. 4C).

3.5. Ca^{2+} mobilization by hypothermal treatment

Cell activation following hypothermal treatment was also investigated by monitoring the intracellular free Ca^{2+} concentration with Calcium Green-1. Fig. 5 shows the typical results of such a life cell imaging experiment. After hypothermal treatment, we could observe Ca^{2+} oscillations exclusively in cells developing a cap-like structure.

4. Discussion

Exposure to environmental stress, like hypo-/hyperthermia, reactive oxygen species or inflammatory cytokines, represents unavoidable events for almost all cell-types. While this may lead to structural damage such as protein denaturation, it can in the same turn induce a physiological response like cell activation and stress protein synthesis [23]. In this study, we utilized fluorescence microscopy to visualize the earliest morphological events following temperature-induced stimulation of Jurkat E6.1 human lymphocyte cells.

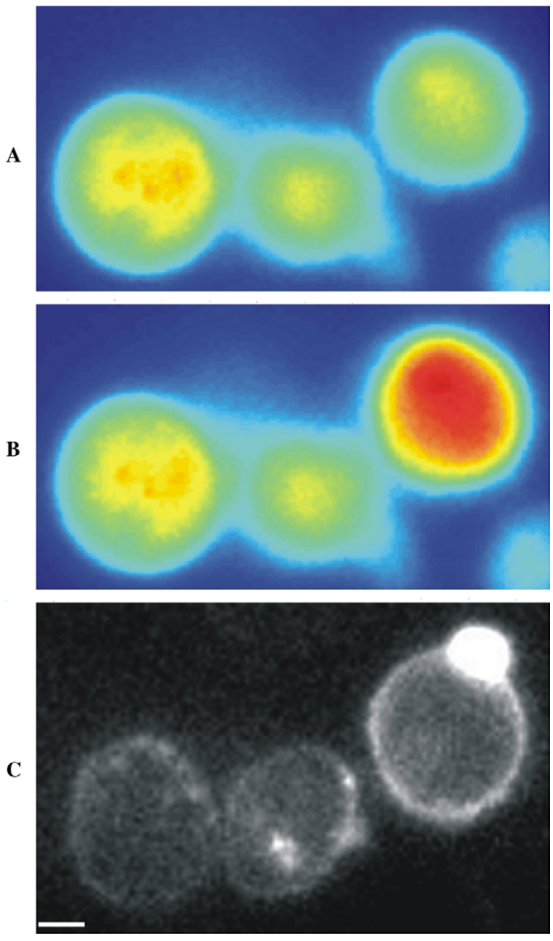

Fig. 5. Ca^{2+} mobilization after hypothermal treatment. Cells were labeled with Calcium Green-1 and anti-CD3 Fab as described in Section 2. Following incubation at 4 °C for 30 min, cells were flushed into a perfusion chamber prewarmed to 37 °C. Ca^{2+} oscillations induced by hypothermal stimulation are shown in (A) and (B), respectively. High Ca^{2+} concentrations are indicated in red. Only cells developing a cap-structure (C) exhibit an increase in intracellular free Ca^{2+} levels (B). Bar, 5 μm.

Plasma membranes of many cell types, including lymphocytes, contain microdomains commonly referred to as lipid rafts. They are biochemically distinct from bulk plasma membrane, are enriched in sphingolipids and cholesterol and contain, e.g., membrane proteins attached to the outer surface via a GPI-moiety or intracellular proteins such as dually acylated Src-family kinase members [24,25] (for review see [26]). To study the distribution of lipid rafts before, during and after hypothermal activation, we used fluorescent labeled CT-B, which binds to the GM1 ganglioside in the outer leaflet of the plasma membrane. Besides being useful markers for lipid-raft membrane microdomains, gangliosides such as GM1 or GM3 have been described in several cell lines to be co-localized with PrP [27–29]. In addition, raft association has been shown to be essential for membrane protein redistribution in polarized T cells [30]. Lipid rafts are shown as small patches scattered randomly all over the surface of unstimulated cells which coalesce into one or a few aggregated patches in response to hypothermal stimulation, a process similar to the actin-mediated reorganization at an immunological synapse following antigen-mediated lymphocyte activation [31]. Formation of such cap-structures was dependent on the temperature-differences used for stimulation with an incubation-shift from 4 to 37 °C yielding the highest degree of activation (see Fig. 1A). First reorganization of plasma membrane domains could be seen after less then 1 min and lasted for approximately 5–10 min (see Fig. 1B). Even hypothermal treatment at 4 °C for only 10 min proved sufficient to detect lymphocyte activation after 2 min at 37 °C (data not shown). Characterization of fully developed cap-structures showed a strict co-localization of CD3 and PrP but not transferrin in GM1-rich domains (see Fig. 2). Although immunoprecipitation experiments performed by our group have not been able to confirm a direct association between CD3 and PrP, recent reports have revealed that PrP is able to form a stable complex with ZAP-70 kinase, a component of the multimolecular TCR-signaling complex formed during T-cell activation [29].

Although much effort has been made to elucidate signal transduction events induced by specific stimulation of lymphocytes via antigen-presenting cells, only few data are available on the initial mechanisms occurring after temperature-induced lymphocyte activation. The most feasible scenario specifies the plasma membrane itself as being the primary sensor for temperature alterations. This could be due to the different fluidic states a membrane acquires at variable temperatures, like rigidification at low temperatures and fluidization during heat-activation (for reviews see [32,33]). A direct influence of changing membrane properties on receptor kinase behavior is conceivable. Signal transduction through the MAPK pathway is usually initiated by ligand-induced receptor dimerization and subsequent auto-phosphorylation. The activation of MAPK (as shown in Fig. 3) and not SAPK/p38 (data not shown) during hypothermal stimulation indicates that we do not induce a classical heat-shock stress-response but rather stimulate lymphocytes via a classical, receptor-clustering route. This might be induced through restricted diffusion of receptor tyrosine kinases in the rigidified plasma membrane increasing or stabilizing potential receptor dimerization/clustering events followed by the subsequent activation of signal transduction cascades. In addition, also a process involving the inactivation of phosphatases seems plausible and awaits further characterization [34].

Hypothermal activation is impaired in T-cells after depletion of membrane cholesterol by MβCD (see Fig. 4A). This treatment was shown to efficiently disturb lipid-raft microdomains thereby interfering with the proper sorting of GPI-anchored proteins [35]. A similar effect was obtained in T-cells treated with Cyt D, an agent which disrupts the actin cytoskeleton [36]. As shown in Fig. 4B, we have been able to abolish formation of GM1-rich structures by this compound. These data indicate that an intact membrane morphology as well as an undamaged cytoskeleton are indispensable for proper lymphocyte activation also in the process of hypothermal stimulation of cells.

Furthermore, temperature-induced stimulation proved sufficient to evoke an increase in intracellular free Ca^{2+} concentrations, one of the obligatory events during T-cell activation (Fig. 5). This corroborates our finding that hypothermal treatment represents indeed a novel route to induce cellular activation in a standardized and accurately described lymphocyte cell line. Current work is in progress to investigate hypothermal treatment in other cells of lymphoid origin as well

as primary lymphocyte cultures to eventually study the physiological relevance of our observations under in vivo conditions.

In summary, our results demonstrate that exposure of Jurkat E6.1 lymphocyte cells to moderate temperature stress leads to a transient activation of the MAPK-signal transduction cascade with concomitant co-localization of CD3 and PrP in GM1-enriched membrane lipid-raft structures. This activation was confirmed by life cell imaging experiments showing Ca^{2+} mobilization following hypothermal treatment. Our results demonstrate for the first time, that hypothermal stimulation is sufficient to trigger the default appearance of an immunological synapse-like structure on a lymphocyte cell line. Co-localization was dependent on the presence of plasma membrane cholesterol and on an intact actin cytoskeleton. Temperature-induced activation persisted for ~10 min and did not lead to the phosphorylation of SAPKs, implying a process different from a conventional heat-shock stress-response.

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