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Effect of temperature on raft-dependent endocytic cluster formation during activation of Jurkat T cells by concanavalin A

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Temperature plays an important role in the immune response. Acclimatization occurs when there are changes in ambient temperature over a long period. In this study, we used the human leukemic Jurkat T cell line to study the effect of temperature on the immune system using concanavalin A (ConA), a plant-derived immunostimulant, as a trigger for T-cell activation. Previously, we have reported endocytic intracellular cluster formation during T-cell activation by ConA with the aid of rafts and polymerization of the cytoskeleton (actin and microtubules). Here, we investigated the effect of temperature on cluster formation (with the aid of three-dimensional images of the cells) and on the stability of rafts, actin, and microtubules. When the temperature was changed between 23°C and 37°C (physiological temperature), clusters could be observed throughout this temperature range. Raft structure was stabilized at lower temperatures but destabilized at higher temperatures. Actin was stable when the temperature was higher than 27°C. When actin was depolymerized, clustering was not observed at 37°C but could be observed at 23°C. There were no changes in microtubules within this temperature range. Thus, raft clustering may be associated with raft stability at lower temperatures (<27°C) and with actin at higher temperatures (≥27°C). Hence, we provided insight into the associations between temperature, rafts, actin, and microtubules in the immune response.

[**Key words:** Temperature; T cell; Rafts; Actin; Concanavalin A]

The response of the human immune system is affected by the ambient temperature. When exposed to cold temperatures, the body attempts to maintain the internal temperature by increasing the production of heat. Longer exposures to low temperatures can cause the body to adapt to the environment, which can affect mRNA expression of immune cell markers as recently reported during cold acclimatization (1). The immune system comprises lymphocytes, one type of which is T cells, which originate in bone marrow and mature in the thymus. T cells can be activated by interacting with antigen-presenting cells (APCs) and T-cell antigen-specific receptor (TCR) molecules. TCR molecules are preclustered at the membrane, they ligates and nucleates signaling molecule which then leads to the activation of T cells (2,3). This immune response takes place in a nanometer-scale gap between T cells and APCs, which is known as the immunological synapse (IS). TCR molecules are concentrated in the raft region of the T-cell membrane at the IS. Raft regions are known to contain many different receptors, including transient receptor potential channels that respond to temperature and ion channels. Previously, raft regions were considered to microdomains of the cell membrane where saturated lipids and cholesterol are concentrated. Lipid rafts are known to have an important role in various cellular functions such as trafficking of lipids, adhesion, migration, growth, and signaling (4,5). Due to the presence of many receptors in rafts, the dynamics of the raft region is considered to be involved in signal transduction. Evidence suggested that there are multiple mechanisms through which rafts affect cell signaling.

Previously, it was shown by Kobayashi et al. (6) that an increase in temperature, which causes a rise in membrane fluidity, induced cluster formation of molecules involved in signal transduction and antigen recognition, which are essential for triggering T-cell activation. Zynda et al. (7) have demonstrated that when T cells are exposed to temperatures of 37.0–39.5°C, co-stimulation of CD4+ T cells via CD28 ligation, which produces interleukin-2, is markedly reduced. On the other hand, these authors showed that a decrease in temperature also resulted into clustering of raft domains in T cells, increased phosphorylation, and activated release of calcium ions. Others have demonstrated that cold induces raft aggregation and T-cell signaling (8). According to a recent study of T-cell activation, temperature decrease was shown to associate with signaling, which is similar to the stimulation achieved by antibodies (9). Therefore, we investigated the critical role of temperature in membrane dynamics.

Cluster formation and activation of T cells depends on the constituents of lipid raft domains, such as cholesterol and sphingomyelin (10,11). In addition, migration, division, and activation of T cells could be responsible for reorganization of the actin cytoskeleton. In our previous and present studies, we used concanavalin A (ConA) to activate T cells. ConA is a mannose/glucose-binding mitogenic lectin that is isolated from jack beans. It exists as a tetrameric molecule consisting of four identical subunits (26.5 kDa each) bearing a carbohydrate-binding site at physiological pH

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(12–14). We have demonstrated that activation of T cells by ConA involves raft-dependent endocytic movement where formation of the vesicles resulted from enlargement of rafts along with actin. These vesicles then move inside the cellular spaces together with microtubules which are radially nucleated from the centrosome. Thus, raft-dependent endocytic movement of vesicles was associated with actin and microtubules (15).

The aim of this study was to examine raft-dependent intracellular cluster formation of Jurkat T cells by ConA activation at different temperatures. We selected the temperature range 23–37°C and focused on the changes in rafts, actin, and microtubules of the cells. Previously, it was shown that interactions between lipid raft domains and cytoskeletal filaments could result in raft clustering and changes in cytoskeletal dynamics (16,17). It is believed that numerous cellular functions may involve interaction between lipid rafts and cytoskeletal components such as actin/myosin or tubulin (18,19).

Here, live Jurkat T cells were stained with cholera toxin B subunit (Ctb), rhodamine phalloidin, and Oregon green 488 to visualize lipid rafts, actin, and microtubules, respectively. Staining enables clear visualization of the changes taking place at different temperatures. It was shown that raft clustering was achieved following the introduction of ConA, where Ctb fluorescence intensity was low near the physiological temperature. Furthermore, raft clustering was stabilized by rafts at lower temperatures (<27°C) and by cytoskeletal actin at higher temperatures (≥27°C), whereas microtubules stabilized raft clustering regardless of the temperature. Thus, we proposed a hypothetical model to explain intracellular cluster formation and changes in raft, actin, and microtubules at these temperatures (23–37°C).

MATERIALS AND METHODS

Materials Roswell Park Memorial Institute 1640 (RPMI1640) medium, rhodamine phalloidin and fetal bovine serum were obtained from Gibco, Thermo Fisher Scientific, Waltham, MA, USA. Oregon Green 488 taxol, and Alexa Fluor 488 (λex= 490 nm, λem= 525 nm) and 555 conjugated cholera toxin B subunit (λex= 555 nm, λem= 565 nm) were obtained from Invitrogen (Eugene, OR, USA). Concanavalin A, Cytochalasin D and bovine serum albumin (BSA) were purchased from Sigma Aldrich, Tokyo, Japan. Phosphate buffer solution (PBS) and actin staining kit wash buffer were purchased from Takara Bio Inc. (Shiga, Japan) and Abcam Japan (Tokyo, Japan), respectively.

Cell culture Jurkat cell line was a kind gift from Riken Cell Bank (Ibaraki, Japan). They were cultured in RPMI 1640 medium. Medium was supplemented with 10% fetal bovine serum at 37°C in a humidified incubator in the presence of 5% CO_2 (20).

Activation of Jurkat cells by addition of ConA Con A identifies glycoproteins in the membrane and α-linked mannose in serum. Activation of Jurkat cells was achieved by the action of ConA (21) at a final concentration of 10 μg/mL (22). ConA (1 μL) was added to the cell suspension at the time of observation.

Staining of lipid raft Cholera toxin B subunit is a fluorescent probe which interacts with monosialoterahexosylganglioside (GM1) and thus used as a label for lipid rafts. Lipid rafts were highlighted with Ctb Alexa Fluor 488 and 555 conjugate (chosen according to the situation) at final concentration of 1 μg/mL. Ctb in PBS was added to cells, blocked with 0.02 % (v/v) BSA in PBS for 30 min on ice and then incubated for 10 min at 37°C.

Staining and depolymerization of cytoskeletal actin Rhodamine phalloidin is a high-affinity filamentous-actin (F-actin) probe conjugated to the red-orange fluorescent dye tetramethyl rhodamine. Rhodamine phalloidin was dissolved in actin staining kit wash buffer at a final concentration of 100 mg/mL and the mixture was used as a stock solution. Cells were washed twice with RPMI 1640 (serum-free) and then stained using 1 mL rhodamine phalloidin stock solution was mixed with 1 mL actin staining buffer. The mixture was kept in the dark for 30 min at 37°C. In order to achieve depolymerization of actin, cells were first treated with 2 μg/mL cytochalasin D (chemical to inhibit the polymerization of actin) in PBS for 10 min at room temperature and then stained using rhodamine phalloidin. Finally, cells were washed twice with actin staining kit wash buffer and dissolved in 1 mL PBS.

Staining of cytoskeletal microtubule Oregon Green 488 paclitaxel was used to stain the microtubules. Stock solution was prepared at 500 mg/mL in PBS. Cells were washed with serum-free RPMI 1640 and then treated with Oregon green 488 for 10 min at 37°C in a humidified incubator in the presence of 5% CO_2. Thereafter, cells were washed twice with PBS.

Microscopic observation of Jurkat cells Live cells were observed using a confocal laser scanning microscope FV 1000-D (Olympus, Tokyo, Japan), 60 × lens, at respective temperatures such as 23°C, 27°C, 30°C, 33°C, and 37°C using thermocontroller (MATS-550RA-BT, Tokai-Hit, Shizuoka, Japan). To process images, Olympus Fluoview and ImageJ software were used. Images are from one representative experiment of three performed.

Quantitative analysis To analyze our data, we considered cells in the range of 100–150. In all the above experiments, we observed and analyzed equal number of cells. As we used fluorescence probes, we made sure that the intensity of the emitting laser stayed constant throughout the experiments.

RESULTS

Effect of temperature on formation of raft-dependent intracellular clusters Raft-dependent intracellular cluster formation is an important indication of T-cell activation by ConA (15). To visualize lipid rafts, we used Ctb (Alexa fluor 488 conjugate), which binds pentavalently to GM1 and this GM1–Ctb complex is a well-known label for lipid rafts. ConA was used to trigger the activation of Jurkat T cells (23). In the absence of ConA, raft-clustering was not observed regardless of the change in temperature (Fig. 1, top images). Previously, we have shown that raft clustering occurs on addition of ConA at 37°C (15). Here, cells were observed in the temperature range of 23–37°C. Minimum

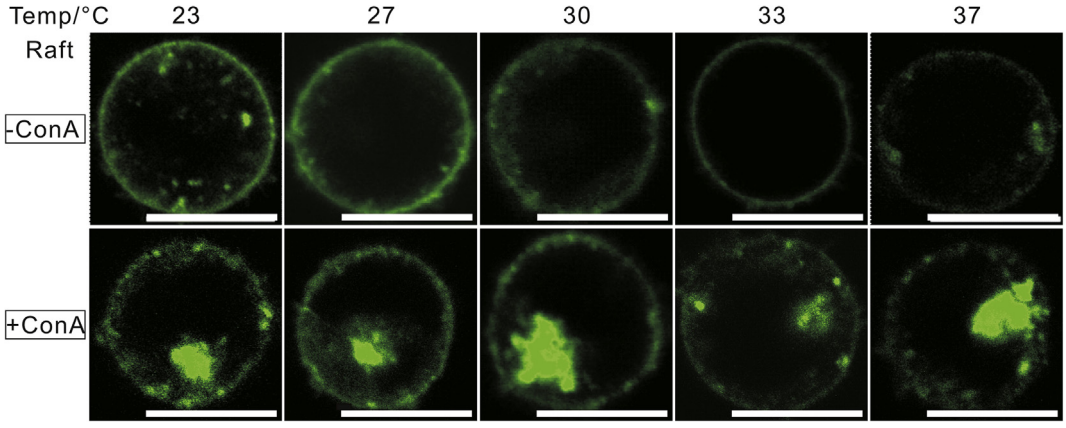

FIG. 1. Influence of concanavalin A (ConA) on raft dynamics. Confocal microscopic images of Jurkat T cells observed at temperatures ranging from 23°C to 37°C. Top images show cells without ConA; bottom images show cells with ConA. Lipid rafts were stained with cholera toxin B subunit (Alexa fluor 488 conjugate; green fluorescence). Images of a representative experiment out of three performed. Number of cells observed was 100–150. Scale bar: 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

temperature was 23°C due to enzymatic activity and thus cell cycle and functions are not feasible at lower temperatures. Ctb fluorescence increased as the temperature was decreased from 37°C to 23°C as shown in Fig. 1. Above 27°C, Ctb fluorescence intensity was very low. This implies that raft stability is increased at lower temperatures (≤27°C) and reduced at higher temperatures (>27°C). Raft stability refers to the stable structure and endocytic movement of rafts and their components. Raft clustering was induced by the addition of ConA at all temperatures. Number of observed cells was in the range of 100–150, of which around 80% of total cells exhibited stability of raft at ≤27°C. Representative images of one of three experiments are shown.

Effect of temperature on polymerized actin Actin is one of the components of the cytoskeleton of the cell. Previously, we reported clustering of actin induced by ConA activation at 37°C (15). In the absence of ConA, fluorescence of rhodamine phalloidin is weak at low temperatures. Here, we investigated cluster formation at different temperatures. By changing the ambient temperature from 23°C to 37°C, we observed changes in actin filaments in T cells (Fig. 2). After adding ConA to the cells, fluorescence intensity was enhanced with increasing temperature and thus the stability of actin increased at higher temperatures. Actin stability can be defined as polymerized and depolymerized state of actin filaments. In other words, it is the transition between globular actin (G-actin) and F-actin. Number of observed cells was in the range of 100–150, of which 82% of total cells showed stability of actin at >27°C.

Intracellular clustering and actin depolymerization Cytochalasin D is an inhibitor of polymerization of cytoskeletal actin. In the absence of ConA, raft clustering was not observed at 23°C or 27°C. We recently reported that raft clustering was not observed when actin was depolymerized at 37°C (15). Thus, we observed cells after actin depolymerization and staining with rhodamine probe only at 23°C and 27°C (Fig. 3). Before activation with ConA, it was not possible to visualize depolymerized actin because the dye intensity was very low at these temperatures. Interestingly, on addition of ConA, clustering was observed at 23°C. In the presence of ConA, raft clustering was not seen at 27°C when actin was depolymerized but actin moved together with rafts at 23°C. Number of observed cells was in the range of 100–150, of which 80% of total cells exhibited actin clusters at 23°C and not at 27°C. During depolymerization, a pool of G-actin forms and it was shown that G-actin is more sensitive to temperature than F-actin (24). Therefore, it is possible that monomeric actin formed by depolymerization traversed with the raft vesicles.

Effect of temperature on cytoskeletal microtubules We investigated the response of microtubules to changing temperature. Oregon green 488 in PBS was used to visualize microtubules. In Fig. 4A and B, red and green fluorescences show rafts and microtubules, respectively. We observed Jurkat T cells at 23°C, 27°C, 30°C, 33°C, and 37°C. First, in the absence of ConA, raft-clustering was not observed and Ctb fluorescence was weak at high temperatures. In the presence of ConA, clustering was dependent on rafts and microtubules. After activation with ConA, clustering of rafts was clearly observed at all temperatures. It is clear from Fig. 4 that microtubules are radially extended from the centrosome (except at 23°C) and raft clustering was clearly observed on microtubules. Thus, we conclude that microtubules are important for raft aggregation and this phenomenon is temperature independent. No. of observed cells were in the range of 100–150, of which 83% of total cells exhibited cluster formation along microtubules. To observe the presence of rafts on centrosomes, we examined the two-dimensional cross-section of cells at different heights (Fig. 4B). These cross-sectional images clearly showed that raft clustering occurred inside and not on the outer surface of cells.

DISCUSSION

Cold acclimatization occurs after long exposure to cold temperatures as a defensive response of the immune system. To sustain the physiological temperature in a cold environment, the sympathetic nervous system must be activated (25). This acclimatization process has been linked to profound alertness against viral infections due to elevated levels of interferons (1,26). Interferons are secreted by lymphocytes in response to the presence of a virus. They behave as mediators to alert the immune system to act against the pathogen. Thus, we investigated the effect of temperature on T cells within the temperature range of 23°C–37°C.

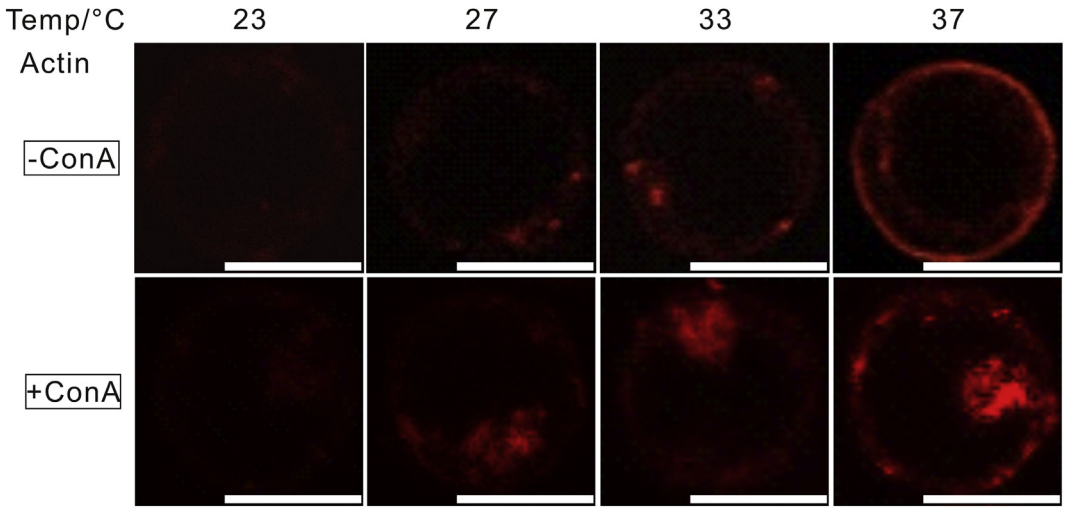

FIG. 2. Effect of temperature on actin in the presence of concanavalin A (ConA). Confocal microscopic images of Jurkat T cells observed at different temperatures (23–37°C). Top images show cells without ConA; bottom images show cells with ConA. Cytoskeletal actin can be visualized with rhodamine phalloidin (red fluorescence). Images of a representative experiment out of three performed. Number of cells observed was 100–150. Scale bar: 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

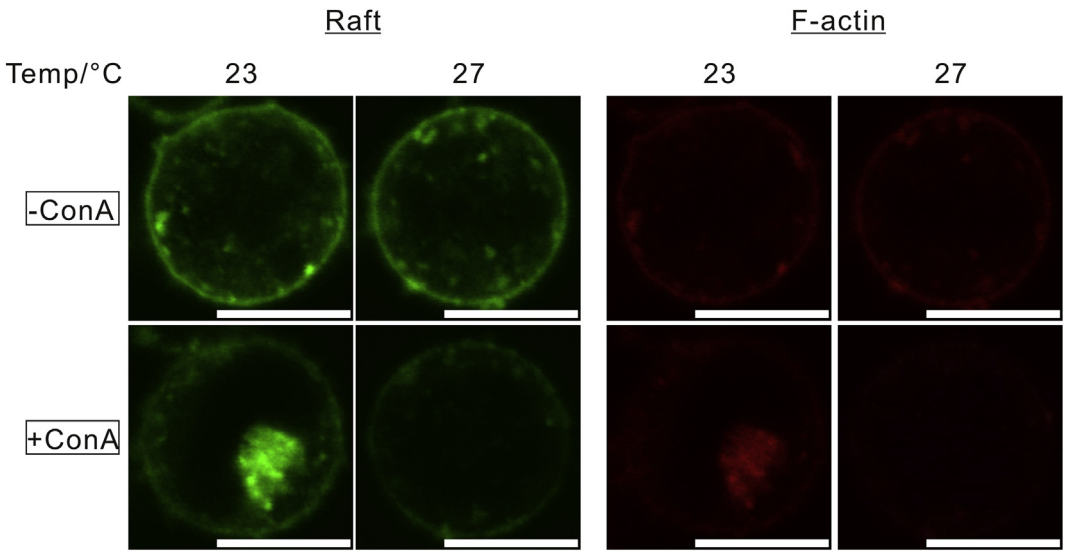

FIG. 3. Effect of temperature on raft- and actin-dependent clustering in depolymerized actin. Confocal microscopic images of Jurkat T cells observed at 23°C and 27°C. Top images show cells without concanavalin A (ConA); bottom images show cells with ConA. Lipid rafts were stained with cholera toxin B subunit (Alexa fluor 488 conjugate; green fluorescence); cytoskeletal actin can be visualized with rhodamine phalloidin and depolymerized with cytochalasin D (red fluorescence). Images of a representative experiment out of three performed. Number of cells observed was 100–150. Scale bar: 10 μm. Filamentous actin (F-actin) is a linear polymer microfilament which is the functional form of actin. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Herein, we have observed raft dynamics by visualizing rafts using fluorescent staining methods at temperatures between 23°C and 37°C. We found that rafts accumulate inside T cells when activated with ConA (Fig. 1). We have previously shown that raft clusters moved from the cell membrane to the cell interior when signaling is triggered by ConA together with actin and rafts through microtubules at 37°C (15). The fluorescent intensity of Ctb, which highlights the raft region, increased as the temperature decreased from 37°C. This increase in marker intensity indicated that raft stability was higher at lower temperatures (23–27°C). Cell-sized liposomes composed of several types of lipids mimicking raft structures exhibited phase separation at low temperature, and the phases are classified as liquid-ordered and liquid-disordered (27). Increased temperature disrupts the heterogeneity and increases the fluidity of the membrane. Bhojoo et al. (28) have demonstrated that raft-like domains are more stable and exhibit higher mechanical forces than non-raft regions of the membrane at 10°C than at room temperature. This suggests rearrangement of membrane lipids to compensate for stress at cold temperatures.

Cytoskeletal actin is an important factor governing stability and function of the raft domain. Cytoskeletal actin affects the lateral distribution of the membrane and protein mobility (29,30). Actin and microtubules are actively involved in signal transduction (31,32). Therefore, we have investigated signal transduction following ConA stimulation by observing the dynamics of rafts and the cytoskeleton (actin and microtubules) at different temperatures. Rhodamine phalloidin (actin) fluorescence intensity was high when the temperature was higher than 27°C. Rosin et al. (33) have demonstrated that polymerization of actin cannot be completed at 10°C as the process is extremely slow. As they increased the temperature from 10°C to 40°C, the rate of actin polymerization increased exponentially. This suggests that actin polymerization is faster at relatively moderate temperatures. In previous experiments with model membranes we showed that liquid-ordered (raft-like) domains disappeared at 27.7°C (34). This suggests that rafts are not stable at physiological temperature and thus clustering will be strengthened by actin at higher temperatures. Actin interacts with the plasma membrane with the aid of membrane protein; phosphatidylinositol 4,5-biphosphate (PIP_2). PIP_2 acts as a co-factor of proteins tethering actin to the plasma membrane, thus coordinates interaction between membranes and cytoskeletal components (35). Though, this is inconsistent with the finding that class I myosins drive motility of actin on binding with PIP_2 (36). Class I myosins act as molecular motors that link actin to membranes. In addition to PIP_2, peripheral myelin protein 22 (PMP22) has been shown to tether actin to plasma membranes (37). But, in the absence of PMP22, the distribution of cholesterol was impaired.

Later, when actin was depolymerized with aid of cytochalasin D, which hinders the formation of F-actin. Wen et al. (38) reported that in the presence of inhibitors of actin polymerization, Ω-profile is maintained, which must be formed during endocytosis. Interestingly, in our study, depolymerized actin was shown to accumulate with rafts at 23°C. It is known that when a vesicle is displaced from the membrane, it can move along actin or actin will come near the surface of vesicles and assist its movement in the cell (39). Thus, we hypothesized that depolymerized actin partially coats raft clusters at 23°C.

However, microtubules have been shown to support clustering at temperatures ranging from 23°C to 37°C. To support the presence of clusters inside cells, we have modeled the two-dimensional cross-section of a single cell (Fig. 5). Previously, we demonstrated that raft accumulation occurred with cytoskeletal actin, which then transferred internally together with radially extending microtubules (15). Our group has also shown that intracellular movement of amyloid beta-42 in T cells depends on the membrane composition, which is responsible for negative curvature (40). These results suggest the involvement of the cytoskeleton in raft dynamics.

Endocytosis can take place when membranes attain sufficient negative curvature to form vesicles. Hirama et al. (41) have recently shown that cholesterol which contains a relatively bulky hydrophobic group induces negative curvature. As a result, cytoskeletal filaments associated with the membrane will be chopped, leading to endocytosis.

We proposed a hypothetical model of raft dynamics at 23°C and 37°C, as shown in Fig. 5. At 23°C, after the addition of ConA, the raft forms a cluster (two-dimensional movement) which later moves inside the cell and is carried along the microtubule (three-dimensional movement). This suggests that intracellular cluster

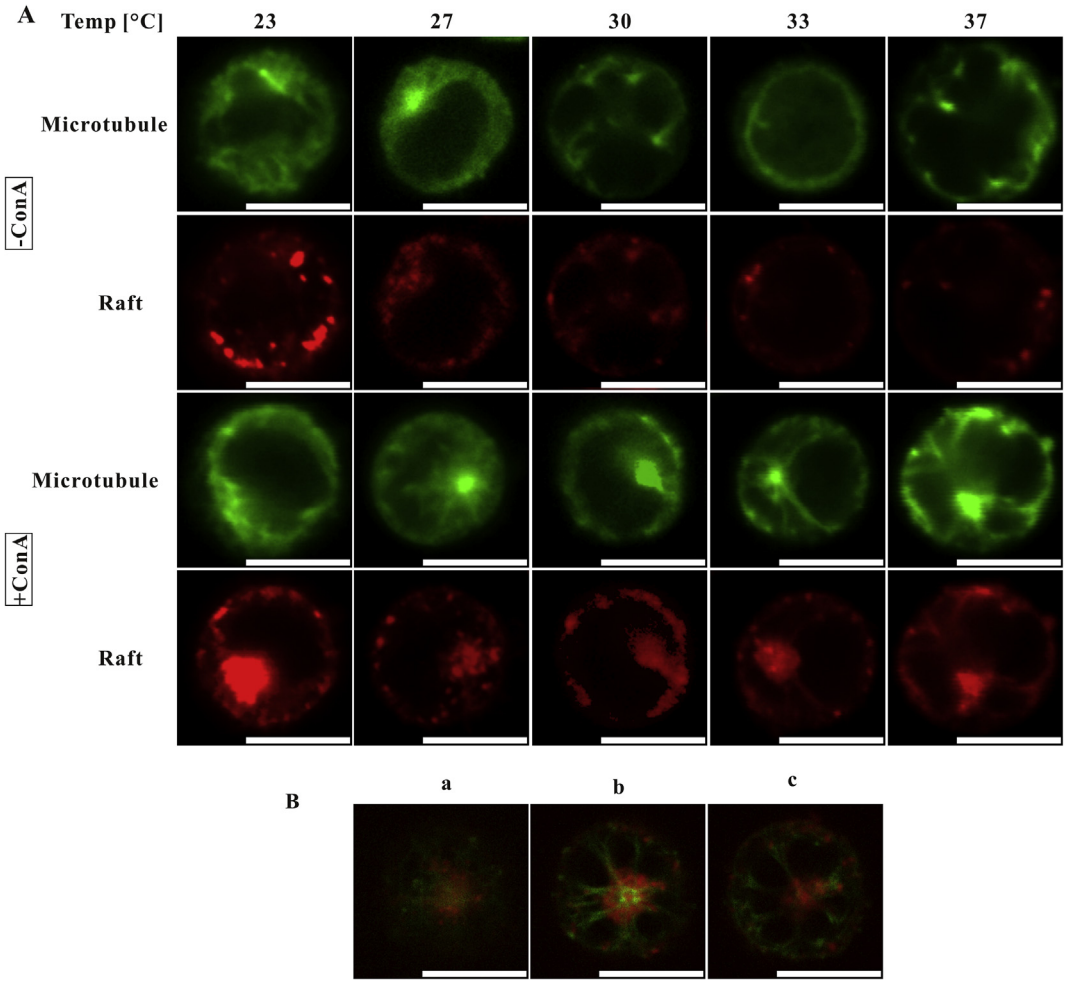

FIG. 4. (A) Effect of temperature on raft- and microtubule-dependent clustering in the presence of concanavalin A (ConA). Confocal microscopic images of Jurkat T cells observed at different temperatures (23–37°C). Top images show cells without ConA; bottom images show cells with ConA. Lipid rafts were stained with cholera toxin B subunit Alexa fluor 555 (red fluorescence); cytoskeletal microtubules were visualized with Oregon green (green fluorescence). (B) Two-dimensional cross-sectional images of a single cell (a–c) at different heights showing the intracellular cluster at the same site as the centrosome. Images of a representative experiment out of three performed. Number of cells observed was 100–150. Scale bar: 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

formation is raft dependent at cold temperatures. However, when actin was depolymerized with cytochalasin D at 23°C, clusters were localized with chopped actin filaments. When the temperature was raised to 37°C, raft clustering was stabilized by cytoskeletal actin which was attached to the cell membrane in this process. T-cell activation with ConA might be achieved by a mechanism other than interaction with APCs. It is important to consider that actin and microtubules play essential roles in the multiple mechanisms of T-cell activation (42). TCR signaling through IS formation is inhibited by actin depolymerization (43). Hence, actin polymerization is important for IS formation via T cell–APC interaction as well as intracellular cluster formation via T cell–ConA interaction. A relationship between movement of the actin cortex and behavior of the plasma membrane has recently been shown using live-cell super-resolution fluorescence microscopy and spatio-temporal image correlation spectroscopy (44). This finding suggested similarity between T-cell activation mechanisms by IS and ConA. Moreover, enlargement of lipid rafts to the size suitable for endocytic vesicular formation was promoted by coordinated functions of both ConA on the surface of the membrane and actin beneath the membrane.

Previously, it was shown that actin polymerization assists clathrin-mediated endocytosis when proteins are not capable of inducing vesicle budding (45,46). This is possible due to coordination between actin and the plasma membrane by proteins that couple these two components at the site of endocytosis (47). During endocytosis, elevated membrane tension can be compensated by the coordination of actin and the plasma membrane (46). Thus, the formation of clusters and their stability depend on rafts and actin at different temperatures. Negative curvature is necessary for vesicle formation which is provided by rafts alone at cold temperatures whereas actin stabilizes and provides negative curvature together with rafts above 27°C.

After raft clustering, signaling platforms were observed as a change in the function of lipid rafts (48,49,50). It was also demonstrated that interactions between lipid rafts and cytoskeletal components are essential for T-cell activation. Previous studies have shown that TCR signaling polymerizes cytoskeletal actin and this eventually causes raft clustering (51,52). As mentioned above, actin, tubulin, and other cytoskeletal proteins can localize to rafts and facilitate communication to the extracellular matrix and provide the basis for cytoskeletal tethering (53). Chichili et al. (54) have shown that as a consequence of actin disruption, raft clustering is abolished. On the other hand, Levitan and Gooch (55) demonstrated that disruption of rafts induced changes in the structure, polymerization, and stability of cytoskeletal components. There have been several studies of raft clustering and the importance of or correlation with cytoskeletal components, but here we intended to investigate the role of temperature on the stability of raft and

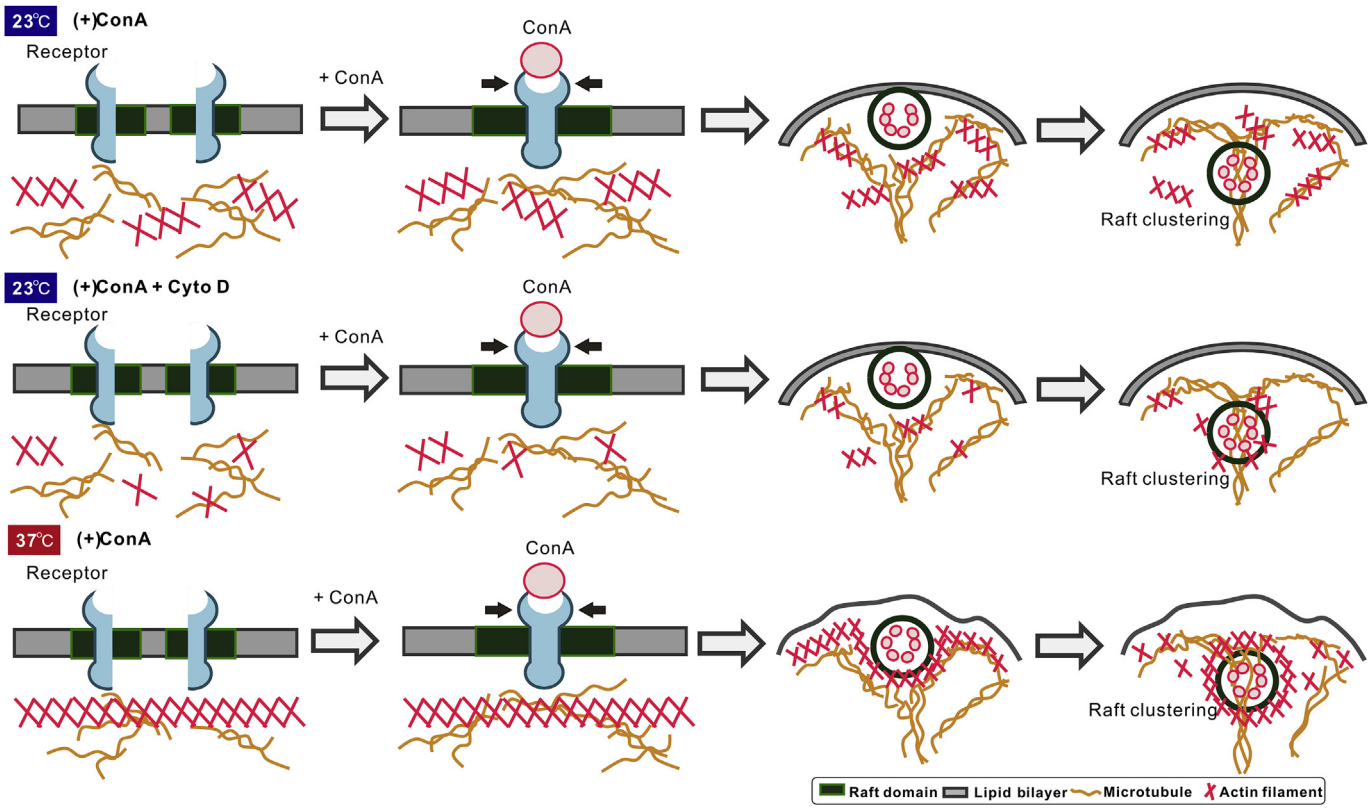

FIG. 5. Model of the influence of concanavalin A (ConA) on raft dynamics at 23°C and 37°C. At 23°C, clustering was raft dependent in the absence of cytochalasin D (Cyto D) while clustering was also strengthened by chopped actin filaments on addition of Cyto D. At 37°C, clustering was actin dependent.

cytoskeletal dynamics. As discussed, temperature plays a crucial role in raft clustering and its stability; the stability was attributed to actin at temperatures above 27°C and to rafts at lower temperatures (<27°C). Raft clustering is microtubule dependent as it can be seen in Fig. 4. Previously we reported that microtubules are important for endocytic movement of vesicle which will not be possible in depolymerized state of microtubules (15). Because raft clustering could be observed even at 23°C, we assume that function and structure of microtubules are not dependent on temperature.

In conclusion, Jurkat T cells are a type of leukemic cell present in the immune system in which temperature has a crucial role. We have demonstrated that raft aggregation was stabilized by actin above 27°C whereas raft-stabilization is actin independent at lower temperatures. Depolymerized actin also assists vesicle movement at cold temperatures. On the other hand, raft accumulation occurs along the microtubules irrespective of temperature. This implies that raft stability is associated with cytoskeletal elements such as actin and microtubules in a temperature-dependent manner. Our findings will be beneficial in the field of cell signaling and provide insight into the role of temperature in immune cell responses.

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