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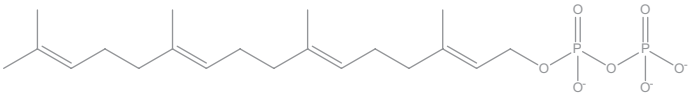
Chapter 5



Fever induces ectopic activation of small GTPases in the autoinflammatory disorder mevalonate kinase deficiency

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In preparation for submission

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ABSTRACT

Mevalonate kinase deficiency (MKD) is an autosomal recessive metabolic and autoinflammatory disorder characterized by life-long episodes of high fever and inflammation. The disorder is caused by mutations in the *MVK* gene encoding the enzyme mevalonate kinase (MK), the first enzyme to follow HMG-CoA reductase in the isoprenoid biosynthesis pathway. We previously showed that in MKD, in particular, the timely synthesis of nonsterol isoprenoids, including geranylgeranyl pyrophosphate, is compromised, which appears to sensitize MKD patients for developing the inflammatory events. Because small GTPases are highly dependent on geranylgeranylation for their proper signaling function and have been implicated in the regulation of inflammatory processes, we studied the effect of fever on geranylgeranylation, activation and localization of three small Rho-GTPases RhoA, Rac1 and Cdc42 in cells from four different MKD patients and compared this with cells from two healthy control individuals. We showed that exposure of cells to 40°C for 24 h results in markedly increased levels of nonisoprenylated, activated GTPases with an altered subcellular localization in MK-deficient cells but not in control cells. We postulate that such ectopic activation of small GTPases gives rise to inappropriate signalling, which may underlie the inflammatory presentation observed in MKD.

INTRODUCTION

Mevalonate kinase deficiency (MKD) is an autosomal recessive autoinflammatory disorder characterized by recurrent episodes of high fever associated with headache, arthritis, nausea, abdominal pain, diarrhea and skin rash. Clinically, patients may present with two distinct presentations, i.e., the hyper-IgD and periodic fever syndrome (HIDS; MIM# 260920) and mevalonic aciduria (MA; MIM# 251170). Because of the overlap between the two entities, they are currently considered to represent the mild and severe clinical and biochemical ends of the MKD spectrum [1-3]. Patients with the HIDS presentation typically show the recurrent episodes of fever with associated inflammatory symptoms [4], whereas patients with the more severe MA presentation in addition to these episodes show developmental delay, dysmorphic features, ataxia, cerebellar atrophy, and psychomotor retardation and may die in early childhood [5]. The distinction between the two presentations can be readily made by determining the residual MK enzyme activities in patient cells, which range from up to 10% in HIDS, when compared with control cells, to below detection levels in MA.

MKD is caused by mutations in the *MVK* gene encoding mevalonate kinase (MK) the first enzyme to follow the rate-limiting and highly regulated HMG-CoA reductase in the isoprenoid biosynthesis pathway [6;7]. The isoprenoid biosynthesis pathway provides cells with a variety of bioactive molecules, i.e. sterol and nonsterol isoprenoids, which play pivotal roles in a range of essential cellular processes including growth, differentiation, glycosylation, isoprenylation and various signal transduction pathways [8].

Although MKD in principle affects the synthesis of all isoprenoids, patients with MKD still are capable of generating isoprenoid end products. In fact, even though MK enzyme activity is below detection levels in cultured skin fibroblasts from MA patients, the *de novo* biosynthesis of cholesterol and nonsterol isoprenoids can be virtually normal [9]. This is due to an increased activity of HMG-CoA reductase, which compensates for a potentially decreased flux through the isoprenoid biosynthesis pathway by elevating the levels of its product mevalonate [9-11]. However, while this flux may be sufficient under normal conditions, previous studies indicated that the inflammatory phenotype associated with MKD is related to an inability to respond rapidly or adequately to an instant further decrease in the activity of MK [12], which is assumed to result in a temporary shortage of one or more isoprenoid end products involved in the regulation or modulation of an innate immune response. There are strong indications that this shortage involves, in particular, nonsterol isoprenoid end products [9;13;14], including geranylgeranyl pyrophosphate, which is required for isoprenylation of a variety of proteins including small GTPases. Protein isoprenylation involves the posttranslational covalent addition of farnesyl pyrophosphate or geranylgeranyl pyrophosphate to cysteine residues at the carboxy terminus of proteins [8]. Isoprenylation is important for many regulatory proteins such as the small GTPases of the RhoA family, because it enables their localization to membranes where they can interact with and activate downstream effector proteins [15;16]. These small GTPases participate in the regulation of a wide variety of cellular functions, including cell cycle progression, morphology and migration, cytoskeletal function, vesicle trafficking, and gene transcription [8;15;16]. Most small GTPases act as molecular switches through cycling between an active GTP-bound state and an inactive GDP-bound state [17]. The cycling between these two

states is mediated by three sets of adaptor proteins, including guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs). GEFs activate small GTPases by promoting the release of GDP and the binding of GTP, whereas GAPs inactivate small GTPases by increasing their intrinsic GTPase activity, thereby releasing the interaction with downstream effector proteins. GDIs prevent the cytosolic activation of small GTPases through binding with their carboxy terminal geranylgeranyl-groups thereby sequestering them away from down-stream targets [17].
Here we report the effect of fever on localization and activation of three small GTPases, RhoA, Rac1 and Cdc42, in MKD. We found that the elevated temperature induces an altered subcellular distribution of these small GTPases in cells from MKD patients but not in control cells. In addition, the elevated temperature results in a markedly increased activation of these soluble GTPases.

MATERIALS AND METHODS

Cell culture

Primary skin fibroblast cell lines obtained from 2 MKD patients with a HIDS presentation, i.e., MKD-HIDS1 and MKD-HIDS2, 2 MKD patients with an MA presentation, i.e., MKD-MA1 and MKD-MA2, and 2 healthy control individuals, i.e., CTR1 and CTR2, with similar passage numbers (15±3) were cultured in 162 cm^2 flasks in nutrient mixture Ham's F-10 with L-glutamine and 25 mM 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES; Invitrogen, Breda, The Netherlands), supplemented with 10% fetal calf serum (FCS; Invitrogen, Breda, The Netherlands) in a temperature- and humidity-controlled incubator (95% air, 5% CO_2) at 37°C until confluence. Subsequently, the medium was replaced with fresh culture medium and cells were cultured for 24 h at 37°C or 40°C. After this incubation, cells were used directly for the different assays described below.

Activated GTPase pulldown assays using membrane and soluble protein fractions

Fibroblasts were washed three times with ice-cold PBS and harvested by scraping in lysis buffer (50 mM Tris pH 7.4, 100 mM NaCl, 10% glycerol, 2 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptine, 10 µg/ml aprotinin, 1 mM benzamidin, 1 mM DTT, 1 mM vanadate). After harvesting, the lysed cell homogenates were sonicated twice (40 J at 8 W output) followed by protein concentration determination using the Bradford assay (Biorad, Veenendaal, The Netherlands) after which the protein concentration was adjusted to 1 mg/ml with lysis buffer. A small amount of total homogenate was saved for immunoblot analysis. The cell homogenates were subsequently separated into a membrane and soluble protein fraction, as described previously [9], to determine the levels of isoprenylated (i.e. membrane-bound) and nonisoprenylated proteins (i.e. soluble protein fraction). In brief, 900 µg of total protein was used for ultracentrifugation (30 min at 100,000g at 4°C). After centrifugation, the supernatant was transferred to another tube and NP-40 was added to an end concentration of 1%. The pellet was dissolved in lysis buffer containing 1% NP-40 and sonicated twice (40 J at 8 W output). A small amount of the supernatant and pellet fraction was saved for immunoblot analysis. The supernatant (soluble) and pellet

(membrane) fractions were then used for activated RhoA, Rac1 and Cdc42 pulldown assays. To this end, the fractions were incubated for 60 min at 4°C with bacterially produced GST-RBD (Rhotekin) [18] (for RhoA pulldowns) or GST-PAK [19] (for Rac1 and Cdc42 pulldowns) bound to glutathione-agarose beads (Sigma, St. Louis, MO, USA). Subsequently, the beads were washed three times with lysis buffer followed by centrifugation (10 sec at 12,000g). Bound proteins were eluted by boiling in SDS-sample buffer and used for immunoblot analysis.

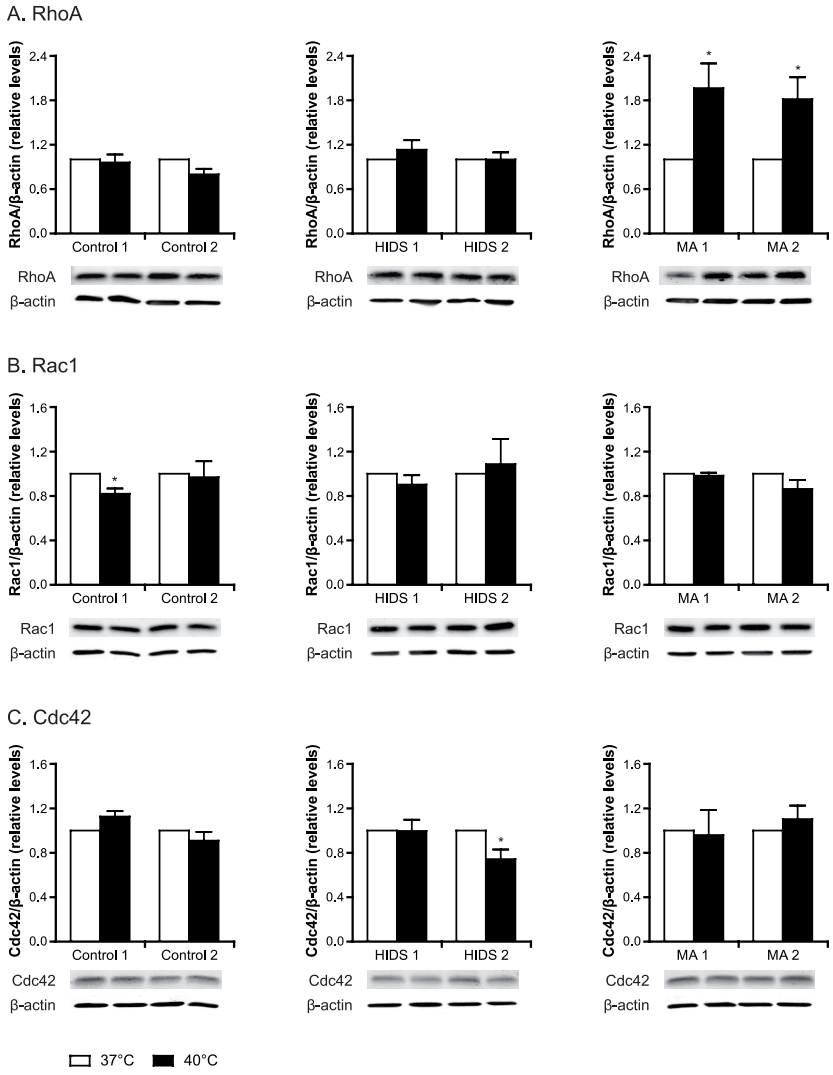

Figure 1. Effect of temperature on expression of RhoA (A), Rac1 (B) and Cdc42 (C) proteins. Control, MKD-HIDS and MKD-MA cells were incubated at 37°C (white bar, set as 100%) and 40°C (black bar) for 24 h. Relative levels of RhoA/β-actin (A), Rac1/β-actin (B) and Cdc42/β-actin (C). Bars show the mean and SEM of three independent experiments. Immunoblots show the results of one representative experiment. Statistic analysis of observed effects was performed with unpaired Student's *t*-test, * = $P < 0.05$, ** = $P < 0.01$.

Immunoblot analysis

Proteins were separated by 12% SDS-PAGE and transferred onto nitrocellulose by semidry blotting. To verify equal transfer of proteins, each blot was reversibly stained with Ponceau S. prior to incubation with antibodies. Membranes were then incubated with a RhoA monoclonal antibody (dilution 1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), a Rac1 monoclonal antibody (dilution 1:10,000; Upstate Biotechnology, Lake Placid, NY, USA), a Cdc42 polyclonal antibody (dilution 1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or a β-actin monoclonal antibody (dilution 1:10,000; Sigma-Aldrich, St. Louis, MO, USA). Antigen-antibody complexes were visualized with IRDye 800CW goat anti-mouse secondary antibody (for monoclonal antibodies) or goat anti-rabbit secondary antibody (for polyclonal antibodies) using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). Densitometric analysis of immunoblots was performed using Advanced Image Data Analyzer (AIDA) software (Raytest, Strauenhardt, Germany).

Statistical analysis

Statistical analysis was performed using unpaired Student's *t*-test. *P* values less than 0.05 were considered significant.

RESULTS

The effect of temperature on the expression of RhoA, Rac1 and Cdc42 proteins

Because many mutations in MKD were shown to have a temperature-sensitive effect on residual MK enzyme activity [12], we previously postulated that an increase in body temperature in MKD patients induces a rapid further decrease in MK activity, which then causes a temporary, instant block of the isoprenoid biosynthesis pathway. As a consequence, the synthesis of down-stream isoprenoids, including geranylgeranyl pyrophosphate required for protein isoprenylation, would be compromised [9;13;14]. To study the effect of elevated temperature on protein isoprenylation, we simulated a fever episode *in vitro* by incubating fibroblasts from MKD patients and control individuals for 24 h at 40°C after which we determined expression, localization and activation of the geranylgeranylated small GTPases RhoA, Rac1 and Cdc42. As shown in figure 1, the increase in temperature had no marked effect on the total cellular levels of these small GTPases, except for RhoA protein in MKD-MA cells, the levels of which were higher than observed in MKD-MA cells cultured at 37°C (Figure 1A).

The effect of temperature on localization of RhoA, Rac1 and Cdc42

To determine the effect of fever on the subcellular localization of the 3 GTPases, i.e., membrane-bound versus soluble fraction, we determined the distribution of the proteins by subjecting cell homogenates to ultracentrifugation. We found that the subcellular distribution of the RhoA (Figure 2A and 2B), Rac1 (Figure 3A and 3B) and Cdc42 (Figure 4A) proteins remained virtually unchanged in control cells cultured at 40°C when compared to culturing at 37°C. In contrast, however, we observed marked changes in the MKD-HIDS and MKD-MA cell lines.

For RhoA, we observed that incubation at 40°C causes a decrease in membrane-bound and a concomitant increase in soluble protein in both the MKD-HIDS and MKD-MA cells

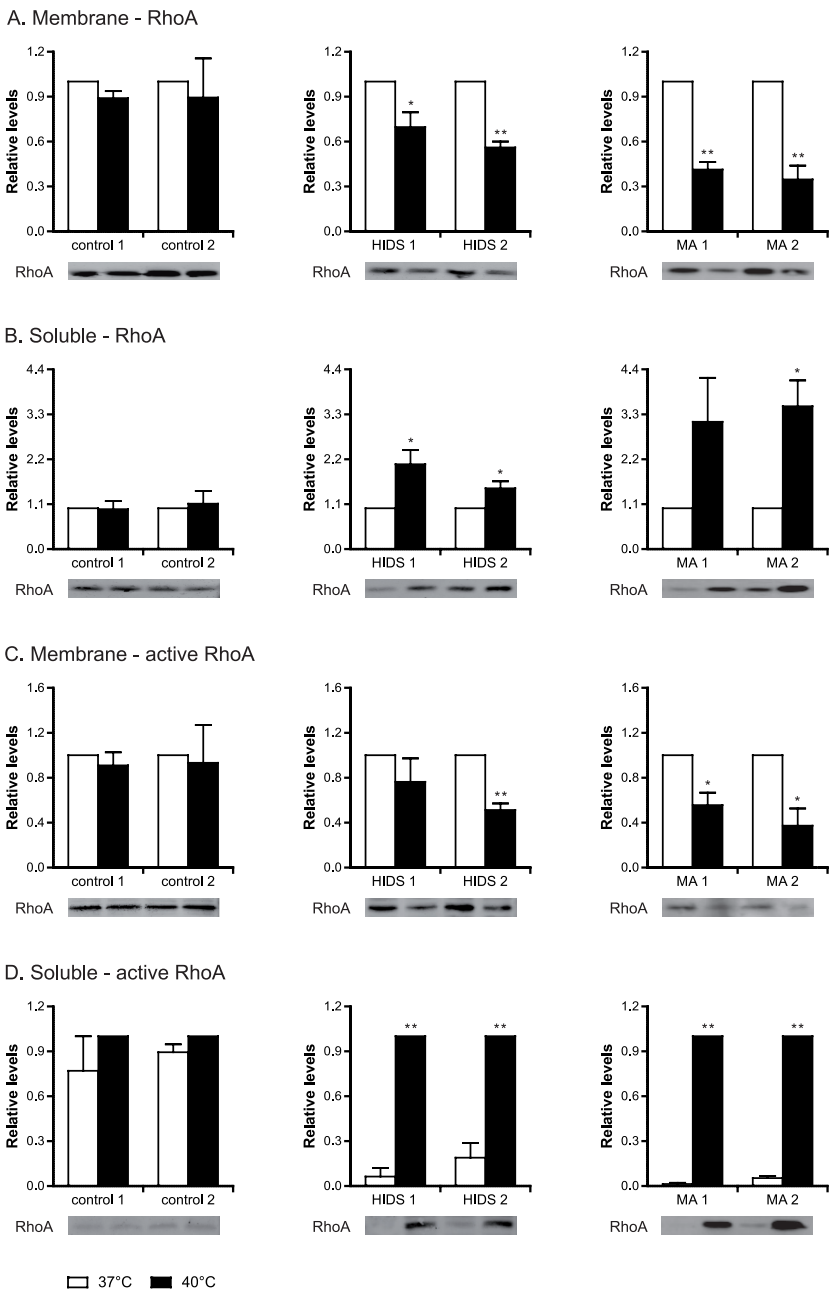

Figure 2. Effect of temperature on localization and activation state of RhoA protein. Control, MKD-HIDS and MKD-MA cells were incubated at 37°C (white bar) and 40°C (black bar) for 24 h. Relative levels of total RhoA (A, white bar set as 100%) and active RhoA (C, white bar set as 100%) associated with membranes. Relative soluble levels of total RhoA (B, white bar set as 100%) and active RhoA (D, black bar set as 100%). Bars show the mean and SEM of three independent experiments. Immunoblots show the results of one representative experiment. Statistic analysis of observed effects was performed with unpaired Student's *t*-test, * = $P < 0.05$, ** = $P < 0.01$.

(Figure 2A and 2B). The effect of temperature is most pronounced in the MKD-MA cells in accordance with the lower residual MK activities in those cells.
The levels of soluble Cdc42 protein are also increased upon incubation at 40°C in the MKD-HIDS and MKD-MA cells (Figure 4A). In contrast to RhoA protein, however, there is no obvious difference in relative increase of soluble Cdc42 protein between the MKD-HIDS and MKD-MA cell lines. The levels of membrane-bound Cdc42 protein appear to be too low to visualize with this assay.
The results for Rac1 protein are less pronounced than for the RhoA and Cdc42 proteins. The levels of membrane-bound and soluble Rac1 protein are decreased and increased, respectively, in MKD-MA cells cultured at 40°C (Figure 3A and 3B), but in MKD-HIDS cells no difference is observed in the levels of membrane-bound and soluble Rac1 protein when the cells are cultured at different temperatures (Figure 3A and 3B).

Effect of temperature on the activation state of soluble and membrane-bound RhoA, Rac1 and Cdc42

To determine if temperature has an effect on activation of small GTPases, we studied the effect of 40°C on the activation state of the soluble and membrane-bound RhoA, Rac1 and Cdc42 protein fractions using pulldown assays specific for activated GTPases. As for the expression levels, we did not observe changes in the activity states of any of the three GTPases when control cells are cultured at 40°C when compared to culturing at 37°C. For the MKD-HIDS and MKD-MA cell lines, however, we noted that culturing at 40°C resulted in a marked increase in activated soluble RhoA protein (Figure 2D) and a decrease of activated membrane-bound RhoA (Figure 2C).
Similar results were observed for activated soluble Cdc42 protein (Figure 4B), the levels of which also become elevated in MKD-MA and MKD-HIDS cells cultured at 40°C.
Culturing at 40°C led to an increase in activated soluble Rac1 protein in the two MKD-MA cells only, albeit to different extents, but not in the MKD-HIDS cells (Figure 3D). Also the decrease in activated membrane-bound Rac1 protein is only observed in the MKD-MA cell lines (Figure 3C).

DISCUSSION

Previously, we postulated that the inflammatory phenotype in MKD can be explained by the fact that in patients with this defect the rate-limiting step determining the flux through the isoprenoid biosynthesis pathway has shifted from HMG-CoA reductase to MK [9], the residual activity of which has become very sensitive to relative minor perturbations, such as (small) rises in temperature [12]. When the flux through the pathway becomes disturbed, this will lead to a shortage of certain nonsterol isoprenoids that are required for proper regulation and/or modulation of the inflammatory response [13;14]. As a consequence, patients will develop a massive inflammatory response to only minor stimuli. Via feedback regulatory mechanisms, this shortage will induce a compensatory increase of HMG-CoA reductase activity [9;12], which leads to re-establishment of the pathway flux and consequently the synthesis of the limiting nonsterol isoprenoid(s), after which the inflammatory response will resolve.
Our previous studies showed that an important nonsterol isoprenoid that becomes limiting in MKD is geranylgeranyl pyrophosphate [14]. Because geranylgeranyl

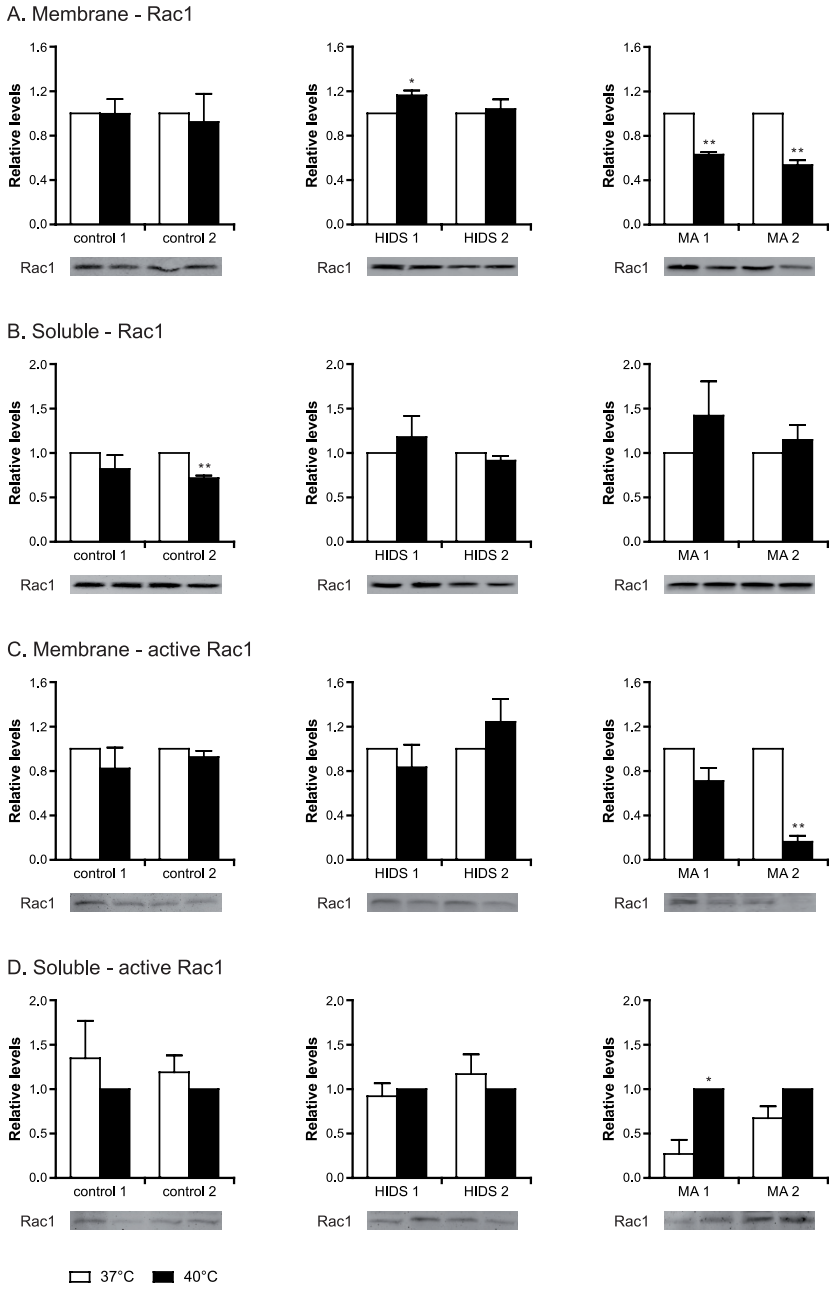

Figure 3. Effect of temperature on localization and activation state of Rac1 protein. Control, MKD-HIDS and MKD-MA cells were incubated at 37°C (white bar) and 40°C (black bar) for 24 h. Relative levels of total Rac1 (A, white bar set as 100%) and active Rac1 (C, white bar set as 100%) associated with membranes. Relative soluble levels of total Rac1 (B, white bar set as 100%) and active Rac1 (D, black bar set as 100%). Bars show the mean and SEM of three independent experiments. Immunoblots show the results of one representative experiment. Statistic analysis of observed effects was performed with unpaired Student's *t*-test, * = $P < 0.05$, ** = $P < 0.01$.

pyrophosphate is required for isoprenylation of proteins such as the small GTPases of the RhoA family to enable their membrane localization and interaction with regulatory proteins [16;17], we expected that depletion of geranylgeranyl pyrophosphate should have an effect on the subcellular location and functioning of such proteins. Indeed, when we incubated fibroblasts of MKD patients with low concentrations of the HMG-CoA reductase inhibitor simvastatin to decrease the pathway flux, we observed that the soluble levels of the RhoA and Rac1 proteins become markedly higher than in similarly treated control cells. In parallel, membrane-bound levels of these GTPases become lower in the MK-deficient cells [9;20].

In the present study, we have studied *in vitro* the effect of fever on localization and functioning of three small GTPases, RhoA, Rac1 and Cdc42. Not only because high fever is the most prominent symptom in MKD, but also because we previously found that a small increase in temperature already results in a rapid further decrease in residual MK enzyme activity in MKD cells and consequently in an instant block in the isoprenoid biosynthesis pathway leading to a shortage of end products [1;12].

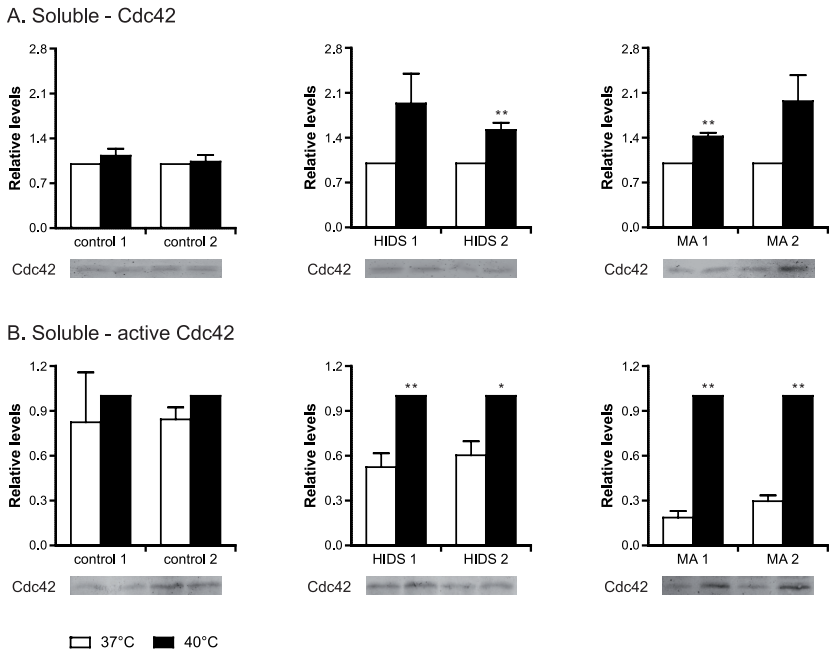

Figure 4. Effect of temperature on localization and activation state of Cdc42 protein. Control, MKD-HIDS and MKD-MA cells were incubated at 37°C (white bar) and 40°C (black bar) for 24 h. Relative soluble levels of total Cdc42 (A, white bar set as 100%) and active Cdc42 (B, black bar set as 100%). Bars show the mean and SEM of three independent experiments. Immunoblots show the results of one representative experiment. Statistic analysis of observed effects was performed with unpaired Student's *t*-test, * = $P < 0.05$, ** = $P < 0.01$.

When we mimicked a fever episode by incubating fibroblasts at 40°C for 24 h, we observed a clear difference between MK-deficient and control cells with respect to the subcellular localization and activation state of RhoA, Rac1 and Cdc42. In general, the high temperature results in a significant increase in soluble levels of activated GTPases in the MK-deficient cells, while in control cells the elevated temperature does not have this effect. As expected, this effect was more pronounced in the MKD-MA cell lines, which have a much lower residual MK activity than MKD-HIDS fibroblasts, rendering these cells more sensitive to disturbances of the isoprenoid biosynthesis pathway flux.
Both the increased levels and activation of the soluble fraction of the GTPases can be explained by a lack of geranylgeranylation, which compromises the membrane-association of the proteins as well as their interaction with GDI proteins, two events that both are mediated by the geranylgeranyl moieties [16;17]. Under normal conditions, binding of GTPases to GDI proteins prevents GTPases to become activated in the cytosol by means of intrinsic nucleotide exchange and promotes the appropriate interaction with specific effector proteins [17]. However, because nonisoprenylated GTPases are not able to bind GDI proteins, the intrinsic nucleotide exchange will lead to cytosolic activation of the GTPases since intracellular GTP levels are higher than GDP levels [21]. Accordingly, nonisoprenylated GTPases will accumulate in the GTP-bound, active state. Previous studies on protein isoprenylation using inhibitors of the isoprenoid biosynthesis pathway in different cell lines showed effects that differ from the effects observed here in cells with genetic MK deficiency. For example, when THP-1 cells [22] and fibroblasts [23] are incubated with simvastatin this results in a marked increase in active soluble Rac1, while the effect of high temperature in MK-deficient cells is more pronounced for RhoA and Cdc42 than for Rac1. This indicates that different disturbances of the isoprenoid biosynthesis pathway may lead to different down-stream effects. Moreover, some studies with statins, bisphosphonates and GGTIs showed an activation of RhoA, Rac1 and Cdc42 proteins [24-26], whereas other studies showed that inhibitors of isoprenylation negatively regulate GTP loading of small GTPases [27-30]. For the different effects seen with statin treatment, however, it cannot be excluded that this is due to pharmacological off-target effects since statins have been reported to induce both anti-inflammatory and pro-inflammatory effects [31-35]. Indeed, statins are commonly used drugs to treat hypercholesterolemia, but are not documented to sensitize treated patients for developing fever and inflammatory responses, indicating that the effect exerted by statin treatment may differ from the effect resulting from a genetic MK deficiency [36].
To conclude, we believe that the ectopic subcellular localization of activated small GTPases may be an, if not the most, important factor underlying the inflammatory phenotype observed in MK deficiency. We postulate that the altered subcellular distribution of activated GTPases, as we demonstrated for RhoA, Rac1 and Cdc42, will lead to inappropriate signalling. This may be failure to induce certain signalling pathways or incorrect induction of other signalling pathways involved in the regulation of the inflammatory response or both. To get insight into which isoprenylated GTPase(s) are affected and causing defective signalling in MK deficiency, we will perform both transcriptome and phosphoproteome profiling of MK-deficient cells. The combined results of these studies should point out which signalling pathways are altered in MKD and thus may unveil novel regulatory mechanisms involved in the innate immune response.

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