SHORT COMMUNICATION

Mevalonate kinase deficiency leads to decreased prenylation of Rab GTPases

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Mevalonate kinase deficiency (MKD) is caused by mutations in a key enzyme of the mevalonate–cholesterol biosynthesis pathway, leading to recurrent autoinflammatory disease characterised by enhanced release of interleukin-1β (IL-1β). It is currently believed that the inflammatory phenotype of MKD is triggered by temperature-sensitive loss of mevalonate kinase activity and reduced biosynthesis of isoprenoid lipids required for the prenylation of small GTPase proteins. However, previous studies have not clearly shown any change in protein prenylation in patient cells under normal conditions. With lymphoblast cell lines from two compound heterozygous MKD patients, we used a highly sensitive in vitro prenylation assay, together with quantitative mass spectrometry, to reveal a subtle accumulation of unprenylated Rab GTPases in cells cultured for 3 days or more at 40 °C compared with 37 °C. This included a 200% increase in unprenylated Rab7A, Rab14 and Rab1A. Inhibition of sterol regulatory element-binding protein (SREBP) activation by fatostatin led to more pronounced accumulation of unprenylated Rab proteins in MKD cells but not parent cells, suggesting that cultured MKD cells may partially overcome the loss of isoprenoid lipids by SREBP-mediated upregulation of enzymes required for isoprenoid biosynthesis. Furthermore, while inhibition of Rho/Rac/Rap prenylation promoted the release of IL-1β, specific inhibition of Rab prenylation by NE10790 had no effect in human peripheral blood mononuclear cells or human THP-1 monocytic cells. These studies demonstrate for the first time that mutations in mevalonate kinase can lead to a milder, temperature-induced defect in the prenylation of small GTPases, but that loss of unprenylated Rab GTPases is not the cause of enhanced IL-1β release in MKD.

INTRODUCTION

Mevalonate kinase deficiency (MKD) is an autosomal recessive, autoinflammatory disorder caused by bi-allelic mutations in the MVK gene that encodes a critical enzyme in cholesterol and isoprenoid lipid biosynthesis.1,2 With the severe form of MKD (mevalonic aciduria; OMIM 251170), patients typically have undetectable levels of mevalonate kinase (MVK) activity and display a variety of neurological and developmental abnormalities.3 The less severe form (hyperimmunoglobulinemia D syndrome or HIDS, OMIM 260920) is characterised by 1–7% normal MVK activity4 and the occurrence of frequent febrile, inflammatory episodes.4 Most patients with MKD are compound heterozygous, commonly with V377I or H20N/P point mutations.4,5

The inflammatory phenotype of MKD appears to be caused principally by loss of synthesis of isoprenoid lipids downstream of MVK, particularly geranylgeranyldiphosphate.7–9 Geranylgeranyldiphosphate is necessary for the post-translational prenylation of proteins of the Rho/Rac/Rap and Rab families of small GTPases. In vitro models of MKD involve treatment of cells with statins (HMG-CoA reductase inhibitor), nitrogen-containing bisphosphonates (inhibitors of farnesyl diphosphate synthase10) or specific inhibitors of geranylgeranyltansferase I such as GGTT-29811 to mimic the block in protein prenylation that is assumed to occur in cells deficient in MVK. These inhibitors predispose cells to increased inflammasome activity and enhanced caspase-1-mediated cleavage of pro-interleukin (IL)-1β9,12–18 a characteristic feature of MKD.

It has been proposed that mutations in MVK may lead to temperature-sensitive changes in folding or stability of the enzyme.5,19 Hence, elevations in body temperature caused by exercise, stress or other triggers could lead to reduced MVK activity and decreased flux through the mevalonate pathway. Inhibition of Rho or Rac prenylation could lead to increased inflammasome activity via

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defects in autophagy.20,21 However, numerous steps in autophagy are also regulated by Rab GTPases, 22 which are prenylated by geranylgeranyltransferase II (GGTase II or Rab GGTase). Despite proposed mechanistic models of enhanced IL-1β release involving decreased Rho/Rac prenylation,21,22 previous studies have failed to demonstrate any intrinsic defect in protein prenylation in cells derived from MKD patients in the absence of statin treatment.23 Furthermore, whether loss of Rab prenylation contributes to the inflammatory phenotype of MKD has not been examined. Here, we took a novel approach, utilising a highly sensitive in vitro prenylation assay,24 to demonstrate for the first time that the prenylation of Rab proteins is altered in cell lines derived from MKD patients following mild heat stress. However, using NE10790 (a specific inhibitor of geranylgeranyltransferase II25–27) we found that the loss of Rab prenylation is not sufficient to enhance the release of IL-1β following lipopolysaccharide (LPS) stimulation of human monocytes.

RESULTS AND DISCUSSION
In this study we specifically sought to answer the long-standing question whether protein prenylation is compromised in MKD cells. We identified two children with MKD. A 3-year-old daughter of unrelated parents (MKD1) was found to be compound heterozygous for missense MVK mutation V377I (c.1129G>A) and c.1058_1060del deletion in exon 11 (a mutation that, to our knowledge, has not previously been reported). She presented with recurrent fever, spastic cerebral palsy of unknown cause, failure to thrive, and elevated IgD and IgA levels (500 mg l−1, 2.74 g l−1, respectively). An 8-year-old boy (MKD2), born to non-consanguineous parents, presented by 12 months of age with 3–4 weekly episodes of periodic fevers lasting 3–4 days in association with abdominal pain, lethargy and arthralgia. His IgA and IgD levels were elevated (9.65 and 1.68 g l−1, respectively). He was confirmed to have compound heterozygous missense mutations in MVK; V377I and H20N (c.58C>A). His parents were confirmed heterozygous carriers.

Epstein-Barr Virus-transformed lymphoblastoid cell lines (EBV-LCLs) were generated using peripheral blood samples from the two MKD patients (MKD1 and MKD2) and from one heterozygous (V377I) parent of each child (Prnt1 and Prnt2, respectively).

MKD patient cells show temperature-dependent accumulation of unprenylated Rab proteins
Previous studies have shown that fibroblasts from MKD patients, including those with V377I/H20P mutations, have an ~50% decrease in MVK activity after culturing for 24 h at 39 °C.19 We used a highly sensitive in vitro prenylation assay with GGTase II24 to determine whether mutations in MVK cause a temperature-dependent accumulation of unprenylated Rab GTPases in EBV-LCLs derived from MKD patients. Lysates from MKD1 and MKD2 cells (patients), and Prnt1 and Prnt2 cells (parents) grown under normal culture conditions (37 °C) showed very low levels of unprenylated Rab proteins.
(Figures 1a and b). However, culturing MKD1 patient cells at 40 °C caused an increase in the presence of unprenylated Rab proteins (a cluster of bands between 21–27 kDa) after 7 days (Figure 1a). Similarly, EBV-LCLs from MKD2 showed an increase in unprenylated Rab proteins when cultured at 40 °C for 3 days and this increased further after 7 days (Figure 1b).

Figure 2 Fatostatin (FAT) decreases SREBP-dependent gene expression and enhances the inhibition of protein prenylation at 40 °C in MKD cells. (a) Schematic diagram of the flux through the mevalonate pathway leading to sterol biosynthesis and protein prenylation. Mutations resulting in reduced MVK activity, as well as pharmacological inhibitors such as statins and nitrogen-containing bisphosphonates (N-BPs), block the production of isoprenoid precursors essential for protein prenylation. The specific inhibitors GGTI-298 and NE10790, respectively, inhibit prenylation of Rho/Rac/Rap GTPases and Rab GTPases. FAT interferes with the upregulation of genes encoding mevalonate pathway enzymes by blocking SREBP-2 activation. (b) TaqMan gene expression analysis of three SREBP-regulated genes in patient (MKD1) and parent (Prt1) EBV-LCLs shows substantial reduction upon FAT treatment. Cells were cultured with dimethyl sulfoxide vehicle (−) or 10 μM FAT (+) at 40 °C for 2 days before harvesting for RNA isolation and real time PCR. dd-CT values are the mean of three separate experiments and expressed relative to the vehicle control (± s.e.m.). (c) FAT decreased the accumulation of unprenylated Rab proteins (uRabs) that occurs in MKD1 (patient) cells after culture for 3 days at 40 °C, but has no effect in Prnt1 (parent) cells. Endogenous 75 kDa biotinylated protein was used as a loading control. The data shown are representative of three independent experiments.

Culturing EBV-LCLs from patients or parents at 40 °C did not affect cell viability (>90% in all cell lines based on trypan blue exclusion) or cell proliferation (based on 5-(and 6)-carboxyfluorescein succinimidyl ester (CFSE) dilution measured by flow cytometry; Supplementary Figure S1). Furthermore, as protein prenylation is a post-translational modification that occurs immediately after synthesis of newly transcribed protein, the accumulation of unprenylated Rab proteins observed in patient EBV-LCLs at 40 °C was not due to a general decrease in cell proliferation, protein synthesis or cell viability. Accumulation of unprenylated Rabs in patient EBV-LCLs was not observed at earlier time points and we could not detect any increase in unprenylated Rap1A (prenylated by GGTTase I) by Western blotting (data not shown), consistent with the far greater sensitivity of the in vitro Rab prenylation assay. To our knowledge, this is the first demonstration that protein prenylation is indeed reduced in cells derived from MKD patients under physiologically relevant conditions that mimic a febrile state.

The accumulation of unprenylated Rab proteins in MKD1 and MKD2 EBV-LCLs cultured for 7 days at 40 °C was comparable to the effect of treating normal EBV-LCLs with 25 μM zoledronic acid, 100 μM NE10790 or 0.1–1 μM simvastatin for 48 h at 37 °C (Figure 1c). This concentration of statin is considerably less than the amount frequently used to inhibit the mevalonate pathway in cell culture models of MKD (10–20 μM), raising the important question whether the effects of such high concentrations of statin in cell culture models are truly representative of changes in prenylation in MKD cells.

Preventing SREBP activation enhances the accumulation of unprenylated Rab proteins in MKD cells

Previous studies have suggested that MKD cells are capable of SREBP transcription factor-mediated upregulation of HMG-CoA reductase and MVK19 and hence normal compensatory changes in the mevalonate pathway in response to lack of cholesterol.5,6,28 This suggests that any lack of protein prenylation in MKD cells would be at least partly overcome by upregulation of enzyme expression (Figure 2a). To examine whether lack of protein prenylation was more pronounced when SREBP-mediated transcriptional upregulation was inhibited, we cultured MKD1 and Prnt1 cells at 40 °C for 3 days in the presence and absence of 10 μM fatostatin, an inhibitor of SREBP cleavage and activation.29 Consistent with an earlier study,29 and in accordance with diminished SREBP-2 transcriptional activity, the expression of the SREBP-2-responsive genes HMG-CoA reductase, MVK and FPP synthase was substantially reduced in the presence of fatostatin in MKD1 and Prnt1 cells (Figure 2b). Although viability remained >96% in both cell lines throughout the experiment, 10 μM fatostatin treatment enhanced the accumulation of unprenylated Rab proteins only in MKD1 but not Prnt1 cells (Figure 2c). Most likely, after fatostatin treatment, the remaining wild-type MVK would be sufficient to maintain adequate flux through the mevalonate pathway and thus maintain normal protein prenylation in Prnt1 cells. However, in MKD1 cells treated with fatostatin, the decreased enzymatic activity of the mutated MVK at 40 °C, together with the inability to replace the defective enzyme pool, results in an even greater reduction in isoprenoid lipids and a more severe loss of protein prenylation.

These data suggest that EBV-LCLs derived from MKD patients (at least those with partial loss-of-function mutations) can overcome the effect of MVK mutations on protein prenylation via mechanisms that include SREBP-mediated upregulation of enzymes of the mevalonate pathway.
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Quantitative SILAC analysis reveals mild accumulation of unprenylated Rab7, Rab14 and Rab1 in MKD cells at 40 °C
To identify and quantify the unprenylated Rab proteins that accumulated in MKD cells at 40 °C versus 37 °C, we enriched the in vitro-prenylated (biotinylated) Rab proteins from lysates prepared from stable isotope labelling with amino acids in cell culture (SILAC)-labelled MKD2 EBV-LCLs cultured either at 37 or 40 °C for 7 days. Enriched, biotinylated Rab proteins were then identified by mass spectrometry and the relative abundance of each was calculated as the fold change between cells cultured at 37 or 40 °C. Ten in vitro-prenylated Rab proteins were identified with a false discovery rate <1% (Table 1). After culture at 40 °C compared with 37 °C, there was an ~200% increase in unprenylated Rab7A, Rab14 and Rab1A, an ~100% increase in unprenylated Rab11A/B, Rab5B and Rab21, and an ~40% increase in Rab6A/B/39A, Rab1B/C and Rab2A/B. Although the fold increase in the unprenylated form of these Rab proteins appears fairly small, the cumulative effect of a small increase in each of these unprenylated proteins, and probably other, less abundant Rab GTPases leads to enhanced IL-1β release.

The increases in unprenylated Rab proteins in MKD2 cells that we identified by SILAC are considerably lower than those observed after treatment of macrophages with very low (nanomolar) concentrations of nitrogen-containing bisphosphonates. This again raises the question whether lack of prenylation of Rab GTPases or Rho/Rac/Rap-family GTPases leads to enhanced IL-1β release by THP-1 monocytes or human PBMC.

Human EBV-LCLs do not release IL-1β. Therefore, to determine whether lack of prenylation of Rab GTPases or Rho/Rac/Rap-family GTPases leads to enhanced IL-1β release, human THP-1 mononcytic cells were treated with simvastatin (prevents prenylation of all small GTPases) or NE10790 (prevents prenylation of Rab GTPases) (Figure 2a). THP-1 cells were pretreated for 24 h with concentrations of each inhibitor that had similar effects on Rab or Rap1A prenylation: 5 μM simvastatin, 10 μM GGTI-298 or 2 mM NE10790 (Figure 3a), before stimulation with 200 ng ml⁻¹ LPS for a further 6 h. These concentrations of inhibitors did not affect cell viability (which was consistently about 80% based on trypan blue exclusion) after 24 h treatment. Although exposure to simvastatin or GGTI-298 significantly enhanced the release of IL-1β caused by LPS stimulation, NE10790 had no effect (Figure 3b). IL-18 release (which, like IL-1β, is inflammasome-mediated) also increased with LPS stimulation, and, similar to IL-1β, LPS-mediated production of IL-18 was doubled by pretreating cells with simvastatin or GGTI-298, but not NE10790 (Supplementary Figure S2). Tumour necrosis factor (TNFα) was not detected in the absence of LPS. Treatment with LPS alone for 6 h caused considerable production of TNFα but neither simvastatin, GGTI-2978 nor NE10790 showed any additional stimulatory effect on TNFα secretion (Supplementary Figure S2). This is consistent with a recent study demonstrating increased IL-1β, but not TNFα, production from MKD versus healthy control peripheral blood mononuclear cells (PBMC) following TLR4 ligation by LPS.

In inhibition of Rab prenylation does not enhance IL-1β release by THP-1 monocytes or human PBMC

To identify and quantify the unprenylated Rab proteins that accumulated in EBV-LCLs from MKD patients, we used stable isotope labelling with amino acids in cell culture (SILAC). Rab21 and Rab23 were identified as the fold change between cells cultured at 37 or 40 °C. Ten Rab proteins were identified: Rab11A/B, Rab14 and Rab1A, an ~100% increase in unprenylated Rab11A/B, Rab5B and Rab21, and an ~40% increase in Rab6A/B/39A, Rab1B/C and Rab2A/B. Although the fold increase in the unprenylated form of these Rab proteins appears fairly small, the cumulative effect of a small increase in each of these unprenylated proteins, and probably other, less abundant Rab GTPases leads to enhanced IL-1β release.

The table below shows the relative abundance of each Rab protein identified in MKD2 cells cultured at 37 or 40 °C.

| Name          | Relative abundance (%) | Ratio 40°C/37°C | Ratio 40°C/37°C |
|---------------|------------------------|----------------|----------------|
| Rab11A/11B    | 8.7                    | 2.1            | 1.8            |
| Rab6A/6B/39A  | 23.4                   | 1.6            | 1.4            |
| Rab1A         | 0.8                    | ND             | 2.9            |
| Rab1B/C       | 12.6                   | 1.6            | 1.5            |
| Rab5C         | 4.6                    | 1.4            | 0.8            |
| Rab7A         | 10.0                   | 3.0            | 2.7            |
| Rab2A/2B      | 5.6                    | 1.4            | 1.4            |
| Rab5B         | 19.8                   | 1.7            | 1.7            |
| Rab14         | 1.0                    | 2.7            | ND             |
| Rab21         | 13.5                   | 2.4            | 1.3            |

In conclusion, our studies demonstrate for the first time that raised temperature causes a mild inhibition of protein prenylation and accumulation of unprenylated Rab GTPases in EBV-LCLs from MKD patients. However, loss of Rab prenylation alone does not lead to enhanced IL-1β release from monocytes following LPS treatment. In contrast, inhibition of prenylation of Rho/Rac/Rap family GTPases is sufficient to predispose monocytes to excessive IL-1β release (Figure 3e); the exact mechanisms involved remain to be definitively proven and validated in MKD cells. Importantly, decreased MVK activity in cultured EBV-LCLs from MKD patients appears to be partially counteracted by increased activity of endogenous SREBP, therefore cultured MKD cell lines may not be a good model to study the functional consequences of human MVK mutations.

**METHODS**

**Cells and reagents**

With Human Research Ethics Committee (HREC) approval, informed consent was obtained prior to obtaining fresh blood samples from the patients and parents. Isolated PBMC were transformed with EBV using standard approaches. EBV-LCLs, THP-1 cells (mycoplasma-free, obtained from ATCC, Manassas, VA, USA) or human PBMC isolated from buffy coat preparations of healthy blood donors were cultured at 37 or 40 °C in RPMI with 10% foetal calf serum. Fatostatin (Tocris, Avonmouth, Bristol, UK), zoledronic acid (a gift from Dr Jonathan Green) and NE10790 (a gift from Dr Hal Ebetino) were dissolved as 40 μM stock solutions in DMSO. Reagents for the prenylation assay were kindly provided by Professor Kirill Alexandrov and Dr Zakir Tnimov (University of Queensland).

**Quantitative PCR analysis**

MKD1 and Prnt cells (5 × 10⁵ cells per ml) were cultured at 40 °C and treated for 2 days with 10 μM fatostatin or dimethyl sulfoxide vehicle before collection for RNA extraction (ISOLATE II RNA Mini Kit, BioLine) and cDNA synthesis (Tetro cDNA Synthesis Kit, BioLine, Alexandria, NSW, Australia) according to the manufacturer’s instructions.
RT-PCR was performed on an ABI Quantstudio7 (Scoresby, VIC, Australia) with specific Taqman primers for HMG-CoA reductase, MVK, FPP and beta-2-microglobulin (B2M) (Thermo Fisher Scientific Scoresby, VIC, Australia, Hs00168352_m1, Hs00176077_m1, Hs01560316_g1 and Hs99999907 respectively). Comparative values of expression (dd-CT) were calculated using beta-2-microglobulin as a housekeeping gene, and presented as relative to the vehicle control.

In vitro prenylation assay

Cells were washed twice with phosphate-buffered saline and lysed by sonication in prenylation buffer (50 mM HEPES, pH 7.2, 50 mM NaCl, 2 mM MgCl₂, 100 μM GDP, 1× Roche complete EDTA-free protease inhibitor cocktail, Basel, Switzerland). Protein was quantified using a BCA assay (Pierce, Scoresby, VIC, Australia). Dithiothreitol (DTT) was added to 50 μg cell lysate to a final concentration of 2 mM, with final concentrations of 2 μM Rab GGTase, 2 μM recombinant Danio rerio Rab escort protein-1, 0.5 μM Biotin-geranyl diphosphate (B-GPP) in a total volume of ~ 50 μl and the reactions incubated for 5 h at room temperature. In vitro-prenylated (that is, biotinylated) Rab proteins were detected on polyvinylidene difluoride blots using streptavidin-680RD (LiCOR, Lincoln, NE, USA). We also consistently detected a narrow doublet (often appearing as a broad singlet) of endogenous biotinylated 75 kDa proteins, which were used as a sample loading control. Blots were also analysed for unprenylated Rap1A using goat anti-Rap1A (sc-1482).

SILAC and mass spectrometry

For quantitative Mass Spectrometry analysis, MKD2 cells were labelled using SILAC for at least 10 cell doublings, as previously described. Cells (10⁶) were seeded into T25 flasks with 8 ml of SILAC medium (Sigma) in duplicate. 'Light'-labelled cells were cultured at 37 °C for 7 days, whereas 'heavy'-labelled cells were cultured at 40 °C for 7 days. The culture medium was supplemented once with 4 ml of fresh medium. Labelling conditions were reversed in a replicate experiment. Cells were washed three times with phosphate-buffered saline and lysed by sonication in prenylation buffer. Protein lysate (840 μg) was mixed in equal proportions from 'light'- and 'heavy'-labelled cells.
and the in vitro prenylation assay performed as described above. In vitro-prenylated proteins were isolated using streptavidin beads, then the bound proteins were analysed on an Orbitrap Velos Pro mass spectrometer (Thermo Scientific, San Jose, CA, USA). The cell pellets were harvested and analysed for unprenylated Rab proteins and Rap1A (as described above) to assess the effect of simvastatin, 10 μM GGTI-298 or 2 mM NE-10790 for 24 h. THP-1 cells were additionally stimulated with 200 ng ml−1 LPS for a further 6 h. Conditioned media were collected and analysed by ELISA for human TNF-α (EisaiK. com), IL-1β, IL-18 (Instant ELISA, eBioscience, San Diego, CA, USA). The cell pellets were harvested and analysed for unprenylated Rab proteins and Rap1A (as described above) to assess the effect of these drug concentrations on protein prenylation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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