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Defective protein prenylation is a diagnostic biomarker of mevalonate kinase deficiency

To the Editor:

Mevalonate kinase (MVK) deficiency (MKD) is a rare, autosomal-recessive autoinflammatory disease that presents in its milder form as hyper-IgD syndrome (HIDS), and in the most severe cases as mevalonic aciduria (MVA).[1] It is widely assumed that the inflammatory symptoms of MKD are caused by defective protein prenylation owing to hypomorphic mutations in MVK. Prenylation is a posttranslational modification of proteins, particularly small GTPases, with isoprenoid lipids that are generated via the mevalonate biosynthetic pathway[2] (Fig 1, *A*). Two recent studies in *Nature Immunology* suggested that loss of the prenylated small GTPases RhoA or K-Ras in MKD results in activation of the pyrin inflammasome and IL-1β secretion.[3,4] However, although MVK is essential for synthesis of the isoprenoid lipid geranylgeranyl diphosphate needed for the prenylation of Rab-, Rho-, and other families of small GTPases (Fig 1, *A*), numerous studies have been so far unable to convincingly demonstrate directly that prenylation of small GTPases is actually altered in MKD. To provide this missing mechanistic evidence, we developed a highly sensitive *in vitro* prenylation assay that enables the detection of unprenylated small GTPase proteins in cell lysates.[5,6] The *in vitro* prenylation assay involves the incorporation of a biotinylated isoprenoid lipid into unprenylated proteins in cell lysates when incubated with a recombinant geranylgeranyl transferase (GGTase) enzyme—either GGTase I (that prenylates Rho, Rac, and Rap proteins) or GGTase II (that prenylates Rab proteins). The small GTPases that are prenylated (and thereby biotinylated) in the *in vitro* prenylation reaction can then be detected with streptavidin after blotting onto membranes.[5,6] We also used western blotting to specifically detect unprenylated Rap1A.[5,7] Both these approaches demonstrated a striking accumulation of unprenylated 21- to 27-kDa Rab proteins and unprenylated 21-kDa Rap1A in PBMCs from an 8-year-old boy with HIDS (V377I/H20N genotype), which was absent in either of his heterozygous, healthy parents (Fig 1, *B*). Furthermore, freshly isolated PBMCs from this patient and 2 other patients with HIDS (MKD1, 2, 3 in Fig 1, *C*) who were compound heterozygous or homozygous for the commonest mutation, V377I, all showed an accumulation of unprenylated Rab GTPases. This was not due simply to an overall increase in the level of Rab proteins because western blot analysis of Rab 14 did not show an increase in patients with MKD (see Fig E1 in this article's Online Repository at www.jacionline.org). Consistent with this, we recently used a quantitative proteomic approach to show that the accumulation of unprenylated Rab proteins in HIDS cell lines was not due simply to a larger pool of Rab proteins.[6]

The accumulation of unprenylated Rab proteins seen in MKD PBMCs was absent in healthy controls and patients with other autoinflammatory diseases such as familial Mediterranean fever, cryopyrin-associated periodic syndrome, and TNF receptor–associated periodic syndrome (Fig 1, *C*; genotypes are shown in Table E1 in this article's Online Repository at www.jacionline.org). The defect in protein prenylation in MKD cells was least obvious in cells from a homozygous V377I patient, consistent with the more variable (and sometimes absent) clinical phenotype of these individuals.[8] However, analysis of PBMCs from this patient on a separate blot, using more protein, revealed a subtle but clear accumulation of unprenylated Rab proteins compared with PBMCs from an unaffected control (Fig 1, *D*).

Unprenylated Rap1A, and unprenylated 21-kDa GTPases that are also substrates for GGTase I,[5,6] such as Rho, Rac, and K-Ras, could be clearly detected in only 1 compound heterozygous patient with HIDS (MKD 1 in Fig 1, *C*) and were not observed in PBMCs from healthy controls or from patients with other autoinflammatory diseases (Fig 1, *C*). The detection of unprenylated Rab proteins in MKD PBMCs therefore appears to be a more sensitive indicator of defective prenylation than detection of unprenylated Rap1A or other proteins modified by GGTase I.

To our knowledge, these analyses are the first direct demonstration that protein prenylation is defective in patients with MKD. The accumulation of unprenylated proteins in fresh PBMCs therefore appears to be a useful diagnostic biomarker to distinguish MKD from other childhood autoinflammatory disorders. Importantly, none of the 3 HIDS/MKD patients analyzed (Fig 1, *B-D*) were undergoing an inflammatory flare at the time of blood collection and were not receiving treatment. Furthermore, we did not find any defect in prenylation in PBMCs from patients treated with statins or bisphosphonate (see Fig E2, *A* and *B*, and Table E2 in this article's Online Repository at www.jacionline.org), 2 widely used classes of drug that also inhibit the mevalonate pathway[7] (Fig 1, *B*). Defective protein prenylation therefore appears to be a specific hallmark of MKD.

We also analyzed a panel of cultured EBV-transformed lymphoblast cell lines (LCLs) derived from patients with the severe (MVA) or milder (HIDS) form of MKD. In contrast to fresh PBMCs from patients with HIDS (Fig 1, *B-D*), the MVA LCLs showed little or no defect in the prenylation of Rab GTPases when the cells were cultured at 37°C (Fig 1, *E*). However, when

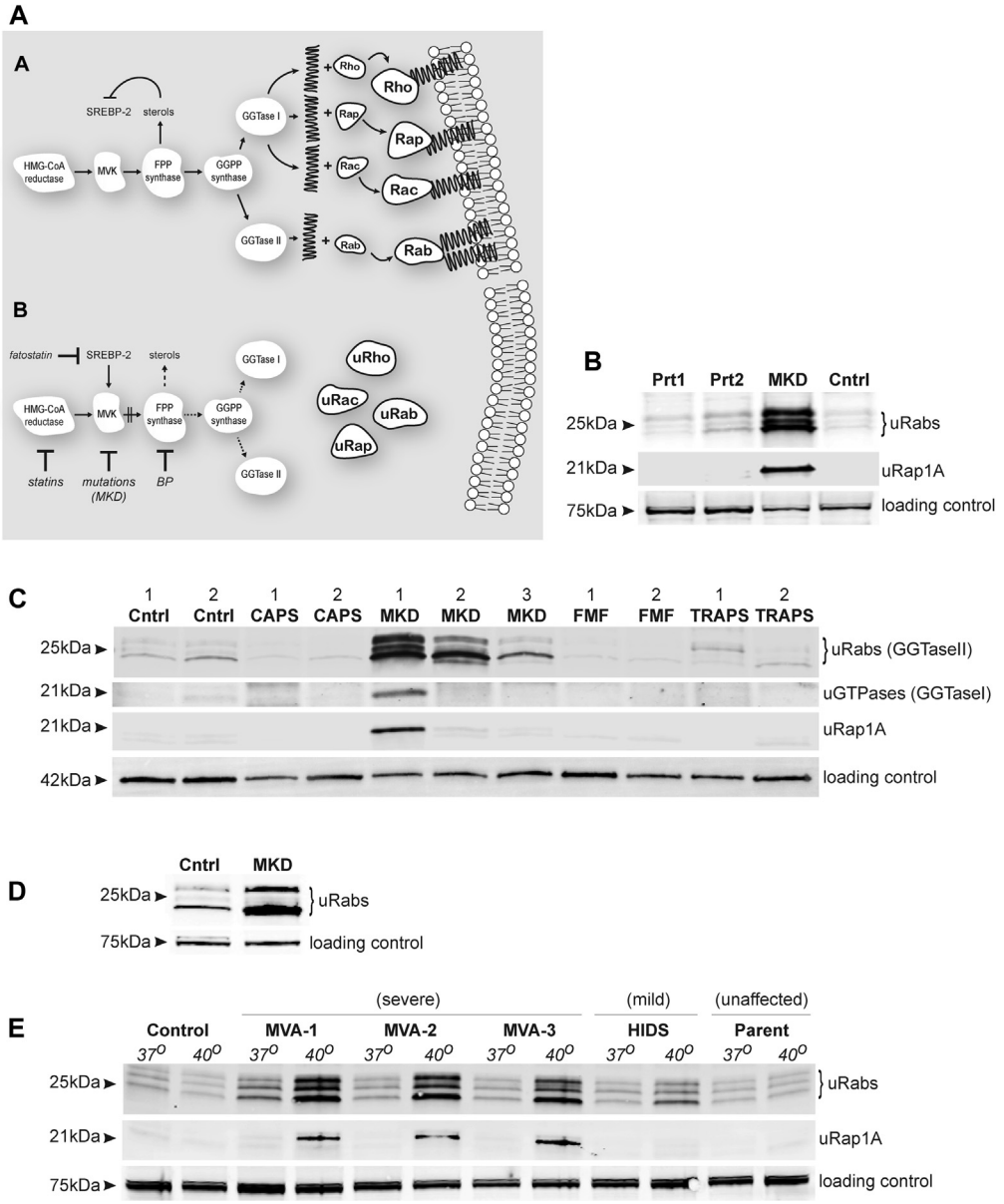

FIG 1. Defective protein prenylation in human PBMCs is a biomarker of MKD. **A,** Schematic diagram of the mevalonate pathway, the production of isoprenoid lipids and protein prenylation. *A:* Isoprenoid lipid tags are irreversibly attached to newly translated GTPase proteins for membrane targeting. GGTase I prenylates Rho-family GTPases (eg, Rho, Rac, and Rap), whereas GGTase II prenylates Rab GTPases. *B:* Detrimental mutations in MVK (or statins and bisphosphonate/BP drugs) decrease the production of the isoprenoid lipid tag (GGPP) required for protein prenylation and membrane localization of small GTPases, leading to accumulation of unprenylated proteins (uRab, uRap, uRho, uRac). Inhibition of SREBP-2 by fatostatin further impairs protein prenylation in cell lines derived from patient with MKD by reducing SREBP-2–mediated upregulation of the defective MVK enzyme. **B,** Accumulation of unprenylated Rab proteins and unprenylated Rap1A is detectable in fresh PBMCs from a boy with MKD (V377I/H20N) but not in PBMCs from either heterozygous parent (Prt1, V377I; Prt2, H20N) or a healthy control (10 μg protein per lane). **C,** Accumulation of unprenylated GGTase I substrates and unprenylated Rap1A, and/or unprenylated Rab proteins, is detectable in fresh PBMCs (4 μg protein per lane) from 3 patients with HIDS (MKD 1, 2, 3) but not in PBMCs from 2 patients each with CAPS, TRAPS, FMF, or 2 healthy controls (Cntrl). Genotypes are shown in Table E1. **D,** Further analysis of MKD 3 (homozygous V377I) in Fig 1, *C*, using 10 μg protein per lane, shows accumulation of unprenylated Rab proteins in PBMCs compared with a healthy control. **E,** Culture of LCLs derived from 3 patients with MVA and 1 patient with HIDS at 40°C for 3 days causes the appearance of unprenylated Rab and Rap1A proteins, but not in LCLs derived from the parent of the patient with HIDS or a healthy control (16 μg protein per lane). *BP*, Bisphosphonate; *CAPS*, cryopyrin-associated periodic syndrome; *FMF*, familial Mediterranean fever; *GGPP*, geranylgeranyl diphosphate; *TRAPS*, TNF receptor–associated periodic syndrome.

cultured at 40°C (to mimic fever) there was a clear accumulation of unprenylated Rab and Rap1A GTPases (Fig 1, *E*), presumably due to misfolding and/or increased degradation of the mutant MVK enzyme.[9] LCLs from a patient with HIDS showed a much milder defect in prenylation at 40°C than did MVA cells, whereas LCLs derived from a heterozygous parent of the patient with HIDS had no defect in prenylation at 37°C or 40°C (Fig 1, *E*). Treatment with fatostatin, a small molecule inhibitor of sterol regulatory element-binding protein (SREBP) function that decreases the expression of *MVK*[6] (see Fig E3, *A*, in this article's Online Repository at www.jacionline.org), enhanced the prenylation defect in MVA LCLs at 37°C and 40°C (Fig E3, *B*). These data, together with our recent observations with LCLs generated from patients with HIDS,[6] strongly suggest that cell lines derived from patients with mutations in *MVK* undergo compensatory changes in the mevalonate pathway in culture, likely involving SREBP-mediated upregulation of *MVK* and other genes of the mevalonate pathway (Fig 1, *A*), and thus are not appropriate models to study the functional effect of mutations in *MVK* on protein prenylation.

In summary, our data provide the long-sought missing evidence for defective protein prenylation in patients with MKD. Furthermore, defective prenylation, particularly of Rab proteins, appears to be a sensitive and specific diagnostic biomarker to distinguish MKD from other autoinflammatory diseases. These observations also pave the way for further studies that will clarify the still poorly understood relationship between genotype and phenotype in MKD.

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Ibrutinib, a Bruton's tyrosine kinase inhibitor used for treatment of lymphoproliferative disorders, eliminates both aeroallergen skin test and basophil activation test reactivity

To the Editor:

Approximately 15 million people, including 8% of children, in the United States have food allergy and are at risk of having life-threatening anaphylactic reactions.[1] There is an unmet need for the prevention of such reactions. Tyrosine kinases, including Bruton's tyrosine kinase (BTK), have been shown to be critical for allergen reactivity by transducing FcεRI crosslinking signals into cellular activation and mediator release from mast cells and basophils.[2] Pharmacyclics, Inc, together with Janssen Pharmaceuticals, recently received Food and Drug Administration approval of ibrutinib (Imbruvica, PCI-32765) as a selective, irreversible BTK inhibitor for the treatment of mantle cell lymphoma, chronic lymphocytic leukemia (CLL), and Waldenstrom's macroglobulinemia. Studies have demonstrated broad and durable activity, with excellent overall tolerability. Regarding tolerability, ibrutinib treatment has been shown to cause a transient increase in blood lymphocytes at the same time as a reduction in lymph node size is typically noted.

METHODS

Cell isolation and culture

Human PBMCs were isolated from buffy coat preparations of fresh blood samples, obtained with informed consent and with approval from the St Vincent's Hospital Human Research Ethics Committee. Cell pellets were snap frozen before analysis. EBV-transformed LCLs were cultured with or without 10 μM fatostatin (Tocris) at 37°C or 40°C in RPMI with 10% FCS. *MVK* mRNA expression was measured by quantitative PCR as previously described.[E1]

In vitro prenylation assay

Cell lysates were prepared as previously described[E1] and 10 to 50 μg of protein were used for *in vitro* prenylation assays with recombinant GGTase I or with GGTase II and REP-1.[E2] *In vitro* prenylated (ie, biotinylated) proteins were detected on polyvinylidene difluoride blots using streptavidin-680RD (LiCOR).[E2] Blots were also analyzed for unprenylated Rap1A using goat anti-Rap1A (Santa Cruz Biotechnology, Dallas, Tex; sc-1482).[E2] Rab14 was detected in 2 μg of PBMC lysates using a 1/200 dilution of rabbit polyclonal anti-Rab14 (H-55, Santa Cruz Biotechnology), 1/5000 peroxidase-conjugated goat anti-rabbit (Pierce), and SuperSignal West Femto ECL reagent (ThermoFisher Scientific, Waltham, Mass). The band of 42-kDa β-actin (detected using 1/5000 mouse mAb 8H10D10, Cell Signalling, Danvers, Mass), or a narrow doublet (often appearing as a broad singlet) of endogenous biotinylated 75-kDa proteins, was used as a sample loading control.

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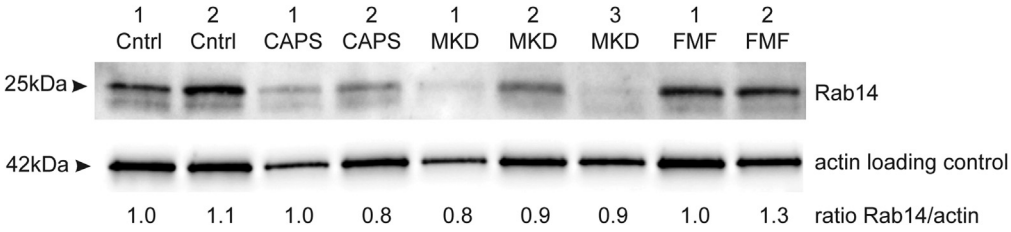

FIG E1. The level of Rab14 is not increased in patients with MKD. Freshly isolated PBMCs from healthy controls (Cntrl) or patients with CAPS, MKD, or FMF (the same patients as shown in Fig 1, *C*, and Table E1) were analyzed by western blotting for Rab14. Endogenous 42-kDa β-actin was used as a loading control. The values correspond to the ratio of the mean intensity of Rab14/β-actin calculated using ImageJ software (NIH, Bethesda, Md), relative to Cntrl1. *CAPS*, Cryopyrin-associated periodic syndrome; *FMF*, familial Mediterranean fever.

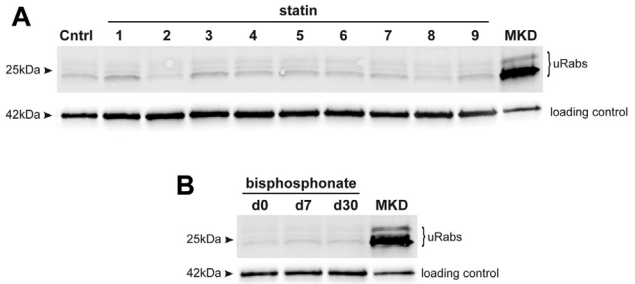

FIG E2. Protein prenylation is defective in PBMCs from patients with MKD but not in PBMCs from statin- or bisphosphonate-treated individuals. **A,** PBMCs were isolated from a healthy control (Cntrl) or from 9 individuals on statin treatment for 0.25 to 20 years (details shown in Table E2). **B,** PBMCs were isolated from a female patient with osteoporosis, at baseline (d0) or at 7 days (d7) and 30 days (d30) after initiating weekly treatment with oral bisphosphonate (Actonel/risedronate). PBMCs from a patient with MKD (compound heterozygous V377I/del) were used as a comparison in Fig E1, *A* and *B*. Cell lysates were analyzed for unprenylated Rab proteins (uRabs) using the *in vitro* prenylation assay (10 μg protein per lane). Endogenous 42-kDa β-actin was used as a loading control.

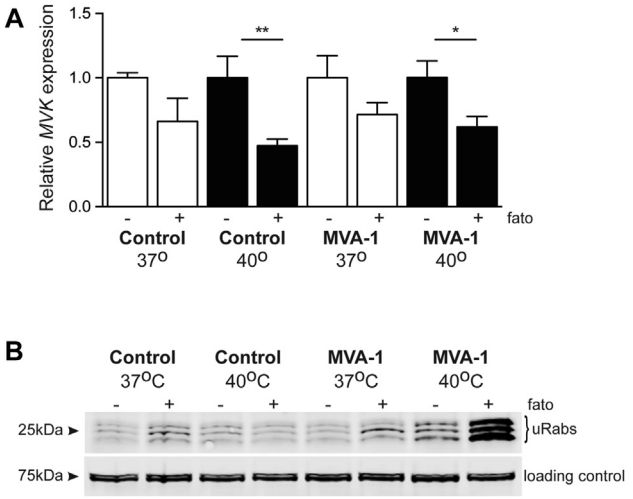

FIG E3. Fatostatin enhances the defect in protein prenylation in a lymphoblast cell line (MVA-1) from a patient with mevalonic aciduria. **A,** Treatment of MVA-1 cells with 10 μM fatostatin for 48 hours at 37°C and 40°C causes a reduction in MVK mRNA expression. $*P < .05$, $**P < .01$, ANOVA with Tukey's multiple comparison test (n = 3 independent experiments). **B,** Culturing MVA-1 cells at 40°C causes a mild accumulation of unprenylated Rab proteins (uRabs) (see also Fig 1, *E*). Treatment of MVA-1 cells with 10 μM fatostatin for 72 hours at 40°C markedly increases the accumulation of unprenylated Rab proteins (35 μg protein per lane). Endogenous 75-kDa biotinylated protein was used as a loading control. The blot shown is representative of 2 independent experiments.

TABLE E1. Genotype of the patients with autoinflammatory disease analyzed in Fig 1, *C*

Patient	Affected gene	Genotype
CAPS 1	NLRP3	het T436I
CAPS 2	NLRP3	het T348M
MKD 1	Mevalonate kinase	V377I/H20N
MKD 2	Mevalonate kinase	V377I/del
MKD 3	Mevalonate kinase	V377I/V377I
FMF 1	Pyrin	het M694V
FMF 2	Pyrin	M694I/E148Q
TRAPS 1	TNF receptor 1	het R121Q (R92Q)
TRAPS 2	TNF receptor 1	het C84Y (C55Y)

CAPS, Cryopyrin-associated periodic syndrome; *FMF*, familial Mediterranean fever; *TRAPS*, TNF receptor–associated periodic syndrome.

TABLE E2. Details of statin treatment for the patients analyzed in Fig E1, *A*

Patient	Age (y)	Sex	Current statin use	Approximate duration of statin treatment (y)
1	82	F	Atorvastatin	10
2	78	F	Rosuvastatin	20
3	71	M	Rosuvastatin	0.25*
4	64	M	Atorvastatin	5
5	74	M	Rosuvastatin	10
6	58	M	Atorvastatin	10
7	71	M	Atorvastatin	14
8	88	F	Atorvastatin	5
9	58	M	Pravastatin	2

F, Female; *M*, male.
*Also receiving prednisone treatment at the time of blood sampling.