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CALR mutant protein rescues the response of MPL p.R464G variant associated with CAMT to eltrombopag.

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Running title

CALR^{del52} rescues MPL^{R464G} response to eltrombopag

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Key points

- 1) Novel MPL^{R464G} variant induces CAMT due to defective trafficking to the cell surface
- 2) CALR mutant renders MPL^{R464G} responsive to eltrombopag

ABSTRACT

Congenital amegakaryocytic thrombocytopenia (CAMT) is a severe inherited thrombocytopenia due to loss-of-function mutations affecting the thrombopoietin (TPO) receptor, MPL. Here, we report a new homozygous MPL variant responsible for CAMT in one consanguineous family. The proband and her sister presented with severe thrombocytopenia associated with mild anemia. NGS revealed the presence of a homozygous *MPL*^{R464G} mutation resulting in a weak cell surface expression of the receptor in platelets. In cell lines, we observed a defect in *MPL*^{R464G} maturation associated to its retention in the endoplasmic reticulum. The low cell surface expression of *MPL*^{R464G} induced very limited signaling with TPO stimulation, leading to survival and reduced proliferation of cells. Overexpression of a myeloproliferative neoplasm-associated calreticulin mutant did not rescue trafficking of *MPL*^{R464G} to the cell surface and did not induce constitutive signaling. However, it unexpectedly restored a normal response to eltrombopag (ELT), but not to TPO. This effect was only partially mimicked by the purified recombinant calreticulin mutant protein. Finally, the endogenous calreticulin mutant was able to restore the megakaryocyte differentiation of patient CD34⁺ cells carrying *MPL*^{R464G} in response to ELT.

INTRODUCTION

Congenital amegakaryocytic thrombocytopenia (CAMT, OMIM 604498) is a rare autosomal recessive bone marrow failure disorder that can evolve into severe aplastic anemia and leukemia.¹ Usually CAMT patients exhibit high thrombopoietin (TPO) serum levels, and a majority of cases carry heterozygous compound or homozygous nonsense, missense and splicing mutations in the *MPL* gene encoding the TPO receptor.^{2,3} The cell surface expression of mature MPL necessary for TPO induced signaling is mainly achieved through the conventional ER-Golgi pathway dependent on MPL association with JAK2 as a chaperone protein.⁴ However, an unconventional, autophagy-dependent manner of delivery of immature MPL to cell surface has also been described.⁵ TPO/MPL signaling controls hematopoietic stem cell survival and megakaryocyte (MK) differentiation.⁶ Interestingly, TPO and MPL play a more important role in humans than in mice. Indeed, *Thpo*⁷ and *Mpl*⁸ knockout mice are viable with a residual platelet production preventing hemorrhage and do not develop bone marrow failure.

MATERIALS AND METHODS

Patients

Blood samples from patients and healthy subjects were collected after informed written consent and obtained in accordance with the Declaration of Helsinki. The study was approved by the Comité de Protection des Personnes CPP N°2020T2-02.

Samples and cell lines

Peripheral blood CD34⁺ cells, megakaryoblastic UT-7 cells, HEK293T cells and Ba/F3 cells were transduced, sorted and cultured as described in the online supplemental methods.

Statistics

Statistical analyses were performed using PRISM software (GraphPad). Statistical significance was determined using a Mann-Whitney test or Two-way Anova with Bonferroni post-test. Differences were considered significant at $p < 0.05$.

More details are in the online supplemental materials and methods.

RESULTS AND DISCUSSION

We report here one French family (**SF1A**) with a novel homozygous *MPL* mutation in two children with severe thrombocytopenia and high TPO levels. The proband is a 3-year-old female (II.3) born to first-cousin parents of Turkish origin and diagnosed with a severe thrombocytopenia (34×10^9 platelets/L) at the age of 3 months. At the age of 2 years, due to the decrease in platelet counts ($< 20 \times 10^9$ /L) associated with a slight anemia, she was first unsuccessfully treated with Romiplostim, then with Eltrombopag (ELT) with only a weak response. Her 8-year-old sister (II.2) was also severely thrombocytopenic (39×10^9 platelets/L) while both parents had normal platelet counts (**SF1B**).

Next generation sequencing identified *MPL* p.R464G (VAF:100%) and *ZFPM1* p.A137G (VAF:50%) variants for the proband and *MPL* p.R464G (VAF:100%) variant for her sister. The *MPL* p.R464G mutation at a VAF of 50% in both parents was confirmed by Sanger sequencing (data not shown). Interestingly, the same variant was recently reported in one unrelated heterozygous *CAMT* patient.⁹ The R464 residue is located on the extracellular domain of *MPL*.

To tease out the mechanism by which this mutant could lead to a severe thrombocytopenia, we first investigated its expression on the surface of platelets from the proband's sister (II.2), presenting higher platelet levels throughout the follow-up. As shown in **Figure 1A**, an almost complete absence of *MPL* at the platelet surface was observed.

Next, we overexpressed the wild-type (WT) and mutant *MPL*^{R464G} in both the human GM-CSF-dependent UT-7 and the IL-3-dependent murine Ba/F3 cell lines. As expected, in UT-7 cells, TPO stimulation of STAT3, STAT5, AKT and ERK pathways was observed only when *MPL*^{WT} was expressed. In both UT-7 and Ba/F3 cells overexpressing *MPL*^{R464G}, TPO induced a slight but significant activation of ERK and a barely detectable phosphorylation of STATs and AKT that were not observed in the absence of cytokines (**Figure 1B, SF2A, B**). No defect in signaling was detected in presence of GM-CSF (**SF2C**). Similarly, a dual luciferase assay in HEK293T cells for STAT5 activity showed a discrete response to TPO (**SF3**), suggesting that the mutation affects either the interaction with TPO or the cell surface expression of *MPL*. Low levels of *MPL*^{R464G} were detected at the cell surface of Ba/F3 cells by flow cytometry (**Figure 1C**), and even lower levels on the membrane of UT-7 cells (not shown). Western blot analysis showed that *MPL*^{R464G} was in an immature, incompletely glycosylated form, but total *MPL*^{R464G} compared to *MPL*^{WT} cell amounts were not different (**Figure 1D**). A defect in trafficking, rather than an increased degradation, was corroborated by the colocalization of *MPL*^{R464G} with the calreticulin ER chaperone, suggesting its retention in the ER (**Figure 1E, SF4**). The

proliferation rate of UT-7 MPL^{R464G} cells in presence of TPO was profoundly decreased compared to the UT-7 MPL^{WT} cells (**Figure 1F**). A complete absence of proliferation was detected in the presence of ELT, a small molecule agonist of MPL that binds in proximity to the mutation¹⁰ (**Figure 1F**). Furthermore, the combination of TPO and ELT did not achieve a significant synergism on proliferation and signaling (**Figure 1F, G, SF5**). Overall, these results indicate that due to a trafficking defect only a small amount of MPL^{R464G} reaches the cell surface inducing a weak signaling in response to TPO stimulation, as previously described for cell lines expressing limited level of MPL^{WT}.¹¹ CAMT can be classified in two groups: type I presents a more severe phenotype and a total loss of cell surface MPL expression due to deletions, nonsense and frameshift mutations, whereas the less severe type II may have residual receptor function due to missense MPL mutations.¹² Our results indicate the MPL^{R464G} variant belongs to the type II CAMT.

Trafficking of the previously described type I CAMT-associated MPL^{R102P} is totally blocked in the ER, however both its traffic and signaling can be restored by the expression of two CALR mutants CALR^{del52} and CALR^{ins5} identified in myeloproliferative neoplasms.¹³ We investigated whether a similar rescue was possible for MPL^{R464G}. As shown in **Figure 2A**, CALR^{WT} overexpression in UT-7 MPL^{R464G} cells was able to induce neither constitutive signaling nor signaling in presence of ELT. In contrast, a slight constitutive signaling was detected with CALR^{del52} overexpression and, unexpectedly, this signaling was enhanced by ELT, but not by TPO (**Figure 2A, SF6**). However, CALR^{del52} was not able to rescue the trafficking defect of MPL^{R464G} (**Figure 2B**). CALR^{del52} has been shown to activate MPL^{WT}¹⁴⁻¹⁶, but only slightly activates MPL^{R464G}. Interestingly, CALR^{del52} renders MPL^{R464G} cells responsive to ELT and increases the response of MPL^{WT} cells to ELT (**Figure 2A, SF6**) suggesting that CALR^{del52} may either induce conformation changes in MPL and a better accessibility of the ELT binding site or/and improve MPL dimerization. In contrast, the interaction between CALR^{del52} and MPL^{R464G} on cell surface may prevent TPO binding and may explain the modest additional effect of TPO on signaling in CALR^{del52}-expressing cells (**Figure 2A**). In agreement with these results, the proliferation of UT-7 MPL^{R464G} cells expressing CALR^{del52} was almost completely restored in presence of ELT, but not of TPO that also did not increase the effects of ELT. This is consistent with our previous findings that CALR^{del52} protein binds to MPL and sterically hinders TPO-binding site¹⁵, although *per se* TPO binding site is not required for the activation by CALR mutants of MPL.¹⁴ In contrast, overexpression of CALR WT elicited a synergism between TPO and ELT (**Figure 2C**). Furthermore, proliferation was completely dependent on JAK2 activation suggesting a normal interaction between MPL^{R464G} and JAK2 (**SF7**). As

CALR^{del52} activates MPL on the cell surface¹³, and since secreted soluble CALR^{del52} was reported to potentially act as a ligand for cell-surface MPL¹⁷- (and manuscript in revision Pecquet et al. 2020), we investigated the effects of exogenously added soluble CALR^{del52} protein on the proliferation of MPL^{R464G}-expressing UT-7 cells. As shown in **Figure 2D**, the soluble CALR^{del52} only partially restored the response to ELT of UT-7 MPL^{R464G} cells suggesting that endogenous CALR^{del52} may bind more efficiently to MPL^{R464G} than exogenous CALR^{del52}. Lastly, we wondered whether CALR^{del52} could restore MK differentiation of patient CD34⁺ cells *in vitro*. To this end, the II.2 patient CD34⁺ cells were transduced with a CALR^{del52} retrovirus and cultured in presence of SCF and ELT (**SF8**). As expected, only the cells expressing CALR^{del52} were able to differentiate into MK (**Figure 2E**).

Overall, these data demonstrate that CALR^{del52}/MPL^{R464G} complex leads to a conformation that is very permissive to the activating effect of ELT, which binds at the outset of the transmembrane domain (H499). CALR^{del52} binds to the extracellular domain of MPL involving glycosylated N117 in D1, likely exposing the H499 containing region where ELT binds. The combination of TPO and ELT seems to have close effect to that observed with exogenous CALR^{del52} and ELT. Taken together, these data may reveal novel approaches to modify the response of MPL to ELT and suggest that a dual activation of MPL may be efficient in cases of low MPL trafficking to the cell membrane.

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Authorship contributions

FBV, GL, LNV designed and performed experiments, analyzed data, contributed to the manuscript draft, MO, PB and NB performed experiments and analyzed data, BN provided clinical and biological follow-up of patients, SC provided bacterial recombinant CALR del52, CM, IP, WV, SC discussed results and contributed to manuscript editing, RF and HR designed and supervised the work and wrote the paper. RF and HR equally contributed to this work. All the authors contributed to the final approval of the manuscript.

Disclosure of Conflicts of Interest

The authors have no competing financial interests to declare.

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Figure Legends

Figure 1. MPL^{R464G} is only weakly expressed on the cell surface and induces a very weak signaling compared to MPL^{WT}.

A. Flow cytometric analyses of MPL expression on platelets from one patient homozygous for MPL^{R464G} (II.2), her heterozygous mother (I.2) and one healthy control (CTR). **B.** Western blot analysis of thrombopoietin (TPO)-induced signaling in UT-7 cells without MPL expression (UT-7), overexpressing wild-type form of MPL (UT-7 MPL^{WT}) and mutant MPL (UT-7 MPL^{R464G}). Cells were starved overnight and stimulated with 10 ng/mL of TPO for 10 or 30 min. No signaling is detected in parental UT-7 cells and a weak ERK signaling is detected in the presence of MPL^{R464G} in UT-7 cells. **C.** Flow cytometric analyses of MPL expression on the surface of Ba/F3 cells overexpressing MPL^{WT} and MPL^{R464G}. **D.** Western blot analysis of the mature (85 kDa) and immature (80 kDa) forms of MPL. The mature form of MPL that is resistant to endoglycosidase H (Endo H) digestion and able to reach the cell membrane is not detectable in UT-7 cells overexpressing MPL^{R464G}. **E.** Immunofluorescence staining for the expression of MPL and calreticulin (CALR) in UT-7 cells overexpressing MPL^{WT} or MPL^{R464G}. The orange arrows indicate MPL colocalization with CALR in the endoplasmic reticulum and the white arrows indicate diffuse cytoplasmic/cell surface MPL expression. Scale bar=30 μ m. In **A** and **C** an anti-MPL coupled with phycoerythrin (PE), in **D** and **E** an uncoupled anti-MPL antibodies were used. **F-G.** Proliferation curves and western blot analysis of signaling of UT-7 cells overexpressing MPL^{WT} and MPL^{R464G} in presence of TPO (10 ng/mL), eltrombopag (ELT, 2 μ g/mL), TPO (10 ng/mL) + ELT (2 μ g/mL), or without cytokines (no cytokines). **F.** 5x10⁴ cells were plated in triplicate in 24-well plates and counted every day for four days. The counts were reported to the condition of UT-7 MPL^{WT}+TPO (in blue color). Shown are averages of three independent experiments at day 4, each performed in triplicate \pm SEM (error bars). *: p<0.05, by Mann-Whitney unpaired nonparametric one-tailed test. **G.** Cells were starved overnight and stimulated for 10 min. A weak ERK signaling is detected in the presence of MPL^{R464G} in all the three conditions (TPO, ELT, TPO+ELT). No signaling is detected in non-stimulated cells (ns).

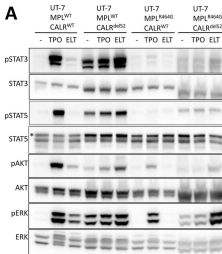
Figure 2. Calreticulin mutant (CALR^{del52}) restores the response of MPL^{R464G} to eltrombopag.

UT-7 and Ba/F3 cells were transduced with retroviruses to overexpress wild-type MPL (MPL^{WT}) or mutant MPL (MPL^{R464G}) together with GFP, and retroviruses overexpressing wild-

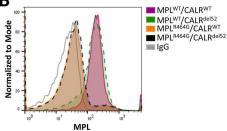
type calreticulin (CALR^{WT}) or mutant CALR (CALR^{del52}) with mCherry, and sorted for GFP⁺mCherry⁺ cells. Parental UT-7 cells were used as negative control. **A.** Western blot analysis of signaling induced by thrombopoietin (TPO) or eltrombopag (ELT). UT-7 cells were cultured in presence of GM-CSF, starved overnight and stimulated with TPO (10 ng/mL) or ELT (2 µg/mL) for 10 min. CALR^{del52} enhances signaling in presence of ELT both in MPL^{WT}- and MPL^{R464G}- expressing cells. **B.** Flow cytometric analysis of MPL expression on Ba/F3 cells using an APC-coupled MPL antibody. CALR^{del52} does not induce MPL^{R464G} traffic to the cell membrane. **C.** Proliferation assay in the presence of thrombopoietin (TPO), eltrombopag (ELT), TPO+ELT or without cytokines (no cytokines). **D.** Proliferation assay in presence of ELT and recombinant CALR^{del52} (recCALR^{del52}) (20 µg/mL). In **C** and **D**, 5x10⁴ UT-7 cells were plated in triplicate in 24-well plates and counted every day for four days. Shown are averages of three independent experiments, each performed in triplicate ± SEM (error bars). In **C**, the counts are reported to the condition of UT-7 MPL^{WT}/CALR^{WT} (in purple color) + TPO and are shown only at day 4 of culture. *p <0.05, Mann-Whitney unpaired nonparametric one-tailed test was used. **E.** Control (CTRL) and patient CD34⁺ cells (II-2) isolated from peripheral blood were transduced with retrovirus encoding CALR^{del52}, sorted at day 2 on CD34⁺ and cultured in presence of SCF and ELT for 12 days. At least 20 mCherry⁺ and 20 mCherry⁻ cells were analyzed. All mCherry⁺ cells expressed MK markers while no mCherry⁻ cells differentiated into MK (not shown). Representative pictures of mCherry⁺ CTRL and II-2 MK (CD41⁺ and vWF⁺) are shown. White arrows indicate individual MK, two different (i and ii) plans of the same view are shown. Scale bar=30 µm.

Figure 2

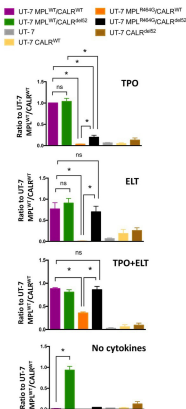
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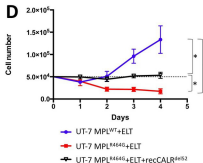
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