From stem cell to platelet
Tijssen, M.R.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Chapter 5

Functional analysis of single amino-acid mutations in the Tpo-receptor c-MPL underlying congenital amegakaryocytic thrombocytopenia

M.R. Tijssen¹, F. di Summa¹, S. van den Oudenrijn¹, J.J. Zwaginga¹,², C.E. van der Schoot¹,³, C. Voermans¹, and M. de Haas

¹Department of Experimental Immunohematology, Sanquin Research, Amsterdam, and Landsteiner Laboratory, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands
²Department of Immunohematology-Bloodtransfusion, Leiden University Medical Centre, Leiden, The Netherlands
³Department of Hematology, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands

Submitted for publication
Summary

Congenital amegakaryocytic thrombocytopenia (CAMT) is a rare disorder that presents with severe thrombocytopenia and absence of megakaryocytes in the bone marrow (BM). The disease may develop into BM aplasia. Genetic defects in the gene encoding the thrombopoietin (Tpo) receptor, c-MPL, are the cause of this disease. In a previous study, we discovered four missense mutations in CAMT patients, predicting Arg102Pro, Pro136His, Arg257Cys, and Pro635Leu. To investigate whether these mutations result in defective Tpo-binding and/or signalling, full-length wildtype and mutant MPL were transduced into K562 cells. Expression levels and ability to activate the MAPK, JAK-STAT and PI3K pathways upon Tpo-binding were studied. Our results predict that MPL carrying the P136H or P635L mutation is not properly expressed, whereas R102P and R257C mutations result in impaired signal transduction. Our results also show that CAMT caused by c-MPL missense mutations can show a severe clinical cause.

Introduction

The thrombopoietin (Tpo) receptor Mpl is expressed on CD34+ hematopoietic progenitor cells (HPCs), megakaryocytes and platelets. Its ligand, Tpo, is the main regulator of megakaryocyte proliferation and differentiation, as reflected by the drastic decrease in platelet numbers both in c-MPL and TPO knock-out mice. Furthermore, c-MPL-deficient mice display hematopoietic stem cell deficiencies, explained by an anti-apoptotic role of Tpo in hematopoiesis.

Signalling via Mpl is thought to be initiated by homodimerization of the receptor upon binding of Tpo. Since the receptor has no intrinsic tyrosine kinase, it regulates the activity of Janus kinases (JAK). After dimerisation the associated JAKs can transphosphorylate each other and a number of other substrates, including Mpl itself. This enables the docking of numerous signalling molecules, including signal transducers and activators of transcription (STATs) which can be phosphorylated by JAKs. Mpl activation can also lead to activation of the mitogen-activated protein kinases (MAPks) extracellular signal-regulated kinases 1 and 2 (ERK1 and 2). Finally, Mpl is able to activate the phosphatidylinositol 3-kinase (PI3K). The exact role of each of these three pathways in proliferation, differentiation and survival signalling in megakaryocytopoiesis is not clear yet. The cellular response is probably a result of activation of, and cross-talk between, different (parts of) pathways.

Congenital amegakaryocytic thrombocytopenia (CAMT) is a rare disorder characterised by an isolated thrombocytopenia and an almost complete absence of megakaryocytes in the bone marrow. CAMT patients often develop complete bone marrow failure. We and others have observed mutations in the c-MPL gene. One of our patients carried two c-MPL alleles with gross abnormalities not compatible with any functional protein. The four other patients carried at least one c-MPL allele with a missense mutation with unclear effects on Mpl function, one of which was shared by two patients (no siblings). Three of these amino-acid substitutions were located in the Tpo-binding domain (R102P, P136H and R257C) and one in the intracellular signalling domain (P635L). In the present study we investigated the functional consequences of these mutations.
Functional analysis of c-MPL mutations

Materials and methods

Patient characteristics
All patients were diagnosed with congenital amegakaryocytic thrombocytopenia and previously described.11,12

Site-directed mutagenesis
Specific point mutations in the full-length human cDNA of c-MPL cloned in pBluescript, were made with a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). All mutations were verified by automated sequence analysis (ABIprim377XL, Perkin Elmer, Norwalk, CT, USA). Nucleotide numbering according to Genbank sequence NM 005373.

Expression of full length c-MPL in K562 cells
Full length c-MPL (mutated and wildtype) was cloned in the LZRSpBMN-linker-IRES-EGFP vector (kind gift of Prof. H. Spits, NKI, Amsterdam, The Netherlands). Retrovirus was produced in a Phoenix packaging cell line (provided by Prof. G. Nolan, Stanford University CA, USA) and used to transduce K562 cells. GFP expressing cells were flow cytometric sorted on a MoFlo flow cytometer (Dako-cytomation, Cambridgeshire, UK). In all experiments >70% of the cells were GFP positive.

Quantitative RT-PCR
Mpl-encoding mRNA levels were quantified with a SYBR Green-based RT-PCR (ABI prism 7000, Applied Biosystems, Foster City, CA, USA). Primers used to amplify a 76-bp fragment of Mpl exon 10 were: forward: 5'-TGGTGACCGCTCTGCATCTA-3' and reverse: 5'-GCAGGAAACTGCCACCTCA-3'. β-glucuronidase (GUS) and EGFP mRNA levels were determined with primers GUS forward, 5'-GAAAATATGTGGTTGGAGAGCTCATT-3'; GUS reverse, 5' CCGAGTAGAGATCCCCCTTTTA 3'; EGFP forward, 5'-AGCAAGACCCCAACGAGAA-3'; EGFP reverse, 5'-GGCGGCGGTCACGAA-3'.

Western blot analysis
The expression of Mpl and GFP protein was detected by 3G4 (anti-Mpl (CD110); 5 μg/mL; kindly provided by Prof. W.G. Kerr, Tampa, FL, USA) and anti-GFP (GFP-20; Sigma-Aldrich, St Louis, MO, USA) on SDS-PAGE and visualized with ELC solution (Amersham Biosciences, Buckinghamshire, UK).

The phosphorylation of signalling molecules after incubation with 50 ng/ml Tpo (Strathmann Biotec AG, Hamburg, Germany) was quantified with ImageJ. Primary antibodies used were Stat3, Phospho-p44/42 MAPK (E10), Phospho-Stat3 (Tyr705), Phospho-Akt (193H12; Cell Signaling Technology, Danvers, MA, USA), anti-Actin (JLA20; Merck, Whitehouse Station, NJ, USA), and ERK-2 (k-23; Santa Cruz Biotechnologies, Santa Cruz, CA, USA).

Cell fractionation
Cells were washed in ice-cold PBS and spun down at 4°C. Cells were resuspended in a hypotonic buffer (30 mM Hapes, 10 mM KCl, 3 mM MgCl₂, 0.2 mM EDTA, 10% glycerol (v/v), 1 mM DTT, and protease inhibitors), followed by incubation in isotonic buffer (similar as the hypotonic buffer, but with 200 mM KCl). Membrane fractions were spun down at 100,000xg for 30 min. After collection of the supernatants...
(cytosolic fraction), the pellets (membrane fractions) were carefully washed with isotonic buffer and resuspended in Western blot sample buffer. Both fractions were analysed on Western blot.

Results

_all mutants, except Mpl-P136H, are expressed by K562 cells_

K562 cells were transduced with a MPL-EGFP construct containing an IRES sequence, hence expressing the Mpl (wildtype or mutant) protein and the GFP protein as separate proteins. Positive cells were selected by flow cytometric sorting on GFP. The level of c-MPL mRNA expression was determined with a real-time PCR (RT-PCR; Table 1). As an mRNA input control, the RT-PCR of the housekeeping gene beta-glucuronidase (GUS) was used. Equal quantities of GUS mRNA and of GFP mRNA were detected in all (transduced) cell lines. The mRNA level of the MPL-P136H mutant was severely decreased compared to transduced wildtype MPL, which suggests instability of the MPL-P136H mRNA. The mRNA level of the other mutants was comparable to that of transduced wildtype MPL.

On Western blot, Mpl-wt protein migrated as two, presumably differently glycosylated, bands (Figure 1). The Mpl-R102P and -R257C mutants showed expression, but only of the lower band. The Mpl-P635L mutant migrated similarly as did Mpl-wt, but less mutant protein was detected. Since the Mpl mRNA levels of this mutant were comparable to that of Mpl-wt, this suggests impaired protein expression, probably because of increased protein degradation. As expected from the RT-PCR data, the Mpl-P136H mutant protein was not detected.

When the cytosol was separated from the membrane compartment, Mpl was detected only in the membrane compartment in all K562 cell lines transduced with (mutated) Mpl, except for P136H (data not shown). This demonstrates that the

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Mpl</th>
<th>GFP</th>
<th>GUS</th>
</tr>
</thead>
<tbody>
<tr>
<td>K562</td>
<td>31.2</td>
<td>35.0</td>
<td>20.1</td>
</tr>
<tr>
<td>K562-Mpl-wt</td>
<td>17.2</td>
<td>16.7</td>
<td>20.0</td>
</tr>
<tr>
<td>K562-Mpl-R102P</td>
<td>16.9</td>
<td>16.5</td>
<td>19.3</td>
</tr>
<tr>
<td>K562-Mpl-P136H</td>
<td>26.0</td>
<td>17.0</td>
<td>20.4</td>
</tr>
<tr>
<td>K562-Mpl-R257C</td>
<td>17.8</td>
<td>17.3</td>
<td>20.4</td>
</tr>
<tr>
<td>K562-Mpl-P635L</td>
<td>16.7</td>
<td>16.7</td>
<td>20.1</td>
</tr>
</tbody>
</table>

Table 1: mRNA levels of mutated and MPL-<i>wt</i> in K562 cells

Mpl mRNA levels were quantified with a SYBR Green-based RT-PCR. Equal quantities of β-glucuronidase and GFP mRNA were detected in all transduced K562 cell lines. The average threshold cycle values of all cell lines, based on two PCR reactions, are depicted. The K562-Mpl-P136H cell line expresses similar amounts of MPL mRNA as did wild-type K562 cells, while mRNA levels of all other transduced mutants are as high as that of transduced MPL-<i>wt</i>, indicating that MPL-P136H mRNA is unstable. Similar results were obtained in a second run with another batch of RNA.
Functional analysis of c-MPL mutations

Figure 1: Protein levels of mutated and Mpl-wt in K562 cells
Protein levels of mutated and Mpl-wt in K562 cells determined by Western blot. When total cell lysates are analyzed K562-Mpl-wt gives two, presumably different glycosylated, bands at the size of Mpl (about 75 kDa), while the R102P and R257C mutant only show the lower band. The P635L mutant protein shows both bands, but is expressed at a lower level than Mpl-wt. As expected from the mRNA analysis, K562-Mpl-P136H shows no Mpl protein expression.

mutated proteins that are expressed can be incorporated in the membrane.

We investigated the surface expression of wt-Mpl and the Mpl mutants with different anti-Mpl monoclonal antibodies (3G4, 5C6, and anti-CD110 [BD]) and flow cytometry; however, we could not detect a specific signal with these antibodies, neither on transfected K562 cells nor on CD34+ haematopoietic progenitor cells, in vitro cultured CD41+ megakaryocytes or the megakaryocytic cell line CHRF (data not shown).

All mutants showed impaired signalling upon Tpo incubation
Signalling via Mpl is thought to be initiated by Tpo-induced homodimerization of the receptor. After activation of Janus kinases (JAKs), subsequently STAT-, MAPK- and the PI3K/Akt-signalling cascades are activated. We observed that all expressed Mpl mutants showed impaired Tpo-driven signalling (Figure 2). The R102P and the P635L mutants did induce phosphorylation of STAT3 and Akt (Figure 2A and B), albeit at a significant lower level than wt Mpl. The level of phosphorylation induced by Mpl-P635L was considerably higher than that induced by Mpl-R102P. Incubation with Tpo did not result in phosphorylation of STAT-3 or Akt via the R257C mutant. The R102P, P635L, and to some extent the R257C mutant were able to induce phosphorylation of ERK, however, the level of phosphorylation was always lower than that induced by Mpl-wt (Figure 2C). In accordance, Ballmaier et al. did not detect Mpl-R102P expression or JAK-2 phosphorylation after treatment with Tpo of platelets from CAMT patients carrying this mutation.13

Incubation with a peptide agonist for Mpl did not result signalling via any of the Mpl mutants
Currently the only treatment for CAMT is allogeneic stem cell transplantation. We tested whether mFc-AMP2 (50 ng/mL; Amgen Inc., Thousand Oaks, CA) was able to activate the mutant receptors. This agonist induced signalling via Mpl-wt, but not via any of the mutated receptors (data not shown).
In this study we investigated whether we could link four different \textit{MPL} missense mutations, identified in patients with CAMT, to their clinical picture. The aim of this study was to investigate whether the mutant Tpo-receptors were still able to interact with Tpo and induce signalling. We observed that the P136H mutant is hardly expressed in a K562 expression model, most probably because of resultant mRNA instability, as has been reported for single nucleotide mutations present in coding regions of other genes.\textsuperscript{20,21} The P635L Mpl mutant showed a low protein expression level, despite an mRNA level comparable to that of Mpl-wt. In agreement with this, we observed reduced signalling by this mutant. It is known that missense mutations can lead to increased protein degradation. However it is difficult to predict the effect of a given mutation on protein folding\textsuperscript{22} and it was beyond the scope of our study.

**Discussion**

In this study we investigated whether we could link four different \textit{MPL} missense mutations, identified in patients with CAMT, to their clinical picture. The aim of this study was to investigate whether the mutant Tpo-receptors were still able to interact with Tpo and induce signalling. We observed that the P136H mutant is hardly expressed in a K562 expression model, most probably because of resultant mRNA instability, as has been reported for single nucleotide mutations present in coding regions of other genes.\textsuperscript{20,21} The P635L Mpl mutant showed a low protein expression level, despite an mRNA level comparable to that of Mpl-wt. In agreement with this, we observed reduced signalling by this mutant. It is known that missense mutations can lead to increased protein degradation. However it is difficult to predict the effect of a given mutation on protein folding\textsuperscript{22} and it was beyond the scope of our study.

**Figure 2:** Phosphorylation of signalling proteins in total cell lysates of K562-Mpl (wt or mutant) after incubation with 50 ng/ml Tpo

Cells were serum starved for the times indicated below and were subsequently incubated with Tpo for 5 minutes. Cell lysates were analysed for the presence of phosphorylated signalling molecules. The amount of staining was analysed with ImageJ. A blot for total protein or actin was used to correct for loading before calculating the amount of phosphorylated protein present (arbitrary units [AU]). Two Western blots were used. On each, Mpl-wt was loaded to be able to correct for differences in protein transfer and development of film. This is a representative experiment of three experiments. **A.** Phospho-Stat3 levels after 4 hours of starvation. **B.** Phospho-Akt levels after 24 hours of starvation. **C.** Phospho-p44/p42 (ERK) levels after 24 hours of starvation.
Functional analysis of c-MPL mutations
to investigate this further. The R102P and R257C mutants showed similar levels of expression, compared to wt Mpl, in the membrane fraction of the K562 cell line. We hypothesized that these mutations, being situated in the extracellular part of Mpl, would interfere directly with Tpo binding. Indeed, triggering of the R102P and R257C mutant with Tpo hardly induced signalling via the JAK, MAPK and PI3K pathway.

Currently, patients with CAMT and development of aplastic anaemia can only be treated with life-saving allogeneic bone-marrow transplantation. With the development of peptide and antibody agonists for the Tpo receptor, another treatment possibility arose. A recent paper describes so-called minibodies, antibody fragments engineered to more efficiently dimerize Mpl. These minibodies appeared to be able to activate the R102P and R257C mutant receptors. While the clinical use of these minibodies is not possible yet, it might be that peptide Mpl agonists which are currently used in clinical trials, might overcome the Tpo-receptor binding defects. In this study, the peptide Mpl agonist mFc-AMP2 did not induce signalling via any of the Mpl mutants, even not the P635L mutant, whereas it did induce signalling via wt-Mpl. The inability to induce signalling via the mutants is probably caused by conformational changes or too low expression levels of the Mpl mutants. Thus, this agonist cannot be used to improve the clinical outcome for CAMT patients carrying the here-described mutants.

Based on the severity of their disease, patients with CAMT have been clinically divided into two groups, with CAMT-I patients having a low platelet count from birth onwards and early progression of the disease into bone marrow failure (median of 22 months) and CAMT-II patients severely thrombocytopenic shortly after birth, but showing a transient increase in platelet counts during early infancy and a delayed progression into bone marrow failure (median of 48 months). By sequence analysis in a total number of 23 patients it was shown that children with CAMT-I carried nonsense mutations leading to complete loss of Mpl protein and CAMT-II patients carried missense mutations leading to amino-acid changes in the extracellular domain of Mpl. Similarly, a late presentation of CAMT was found in three siblings who were compound heterozygotes for an MLP allele carrying a missense mutation and an MPL allele carrying a mutation leading to an mRNA splicing defect.

In the current study, the patients with the missense R102P mutation (patient 1 and 4 in 12) carried a nonsense mutation (Trp491Stop) causing a premature stopcodon or a mutation causing a splice defect 3’ of exon 3 in the other c-MPL allele. The R102P mutant showed some residual Mpl activity in our study, in concordance with the patients’ CAMT-II-like course of disease. Both patients showed low platelet counts from birth onwards. One patient was transplanted before development of anaemia or leukopaenia at the age of two years and four months. The other patient showed some signs of aplasia at the moment of transplantation at the age of 3.5 years.

The patient carrying the P136H mutation (patient 3 in 12) had a frame-shift inducing 7-bp deletion in exon 6 in the other c-MPL allele, resulting in a premature stopcodon at amino-acid position 368 and thus production of dysfunctional protein. Because no expression was found for the P136H mutant, a CAMT-I phenotype was predicted. Indeed, the patient had platelet numbers below 20 x 10^9/L from birth onwards and early development of anaemia and leukopaenia.

The last patient studied carried two c-MPL alleles with missense mutations: R257C and P635L (patient 2 in 12). We observed that signalling via R257C was almost absent and that, even in our expression system, the P635L mutation caused
significantly reduced expression and thereby reduced signalling activity. It may be that in vivo, the P635L mutant will not be expressed. The platelet count of this patient was low at birth and remained low (nadir of 2x 10^9/L at the age of 2 years, pre bone marrow transplantation). This points to a CAMT-I phenotype, in which early progression into bone marrow failure is expected.

In conclusion, our results indicate that missense mutations in c-MPL can also cause reduced protein expression or non-functional protein, leading to a CAMT-I phenotype. Thus, although the type of MPL mutation has a strong influence on the course of disease, molecular investigation of the functionality of the mutant needs to be added to further predict the clinical course.

Acknowledgements
The authors thank Eloise Anthony and Marion Kleijer for help with cloning and construction of the cell lines. The authors also thank Prof. D. Roos for critically reading the manuscript. Dr. M.C.A. Bruin is acknowledged for providing clinical data of the patients.

Reference list
12 van den Oudenrijn S, Bruin M, Folman CC, Bussel J, de Haas M, von dem Borne AE. Three parameters, plasma thrombopoietin levels, plasma glycocalcin levels and megakaryocyte


19 Germeshausen M, Ballmaier M, Welte K. MPL mutations in 23 patients suffering from congenital amegakaryocytic thrombocytopenia: the type of mutation predicts the course of the disease. Hum Mutat 2006; 27: 296.


