Ligand-independent Thrombopoietin Mutant Receptor Requires Cell Surface Localization for Endogenous Activity*

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The activating W515L mutation in the thrombopoietin receptor (MPL) has been identified in primary myelofibrosis and essential thrombocythemia. MPL belongs to a subset of the cytokine receptor superfamily that requires the JAK2 kinase for signaling. We examined whether the ligand-independent MPL_W515L mutant could signal intracellularly. Addition of the endoplasmic reticulum (ER) retention KDEL sequence to the receptor C terminus efficiently locked MPLW515L within its natural ER/Golgi maturation pathway. In contrast to cells expressing the parental MPL_W515L, MPL_W515L-KDEL-expressing FDC-P1 cells were unable to grow autonomously and to produce tumors in nude mice. When observed, tumor nodules resulted from in vivo selection of cells leaking the receptor at their surface. JAK2 co-immunoprecipitated with MPL_W515L-KDEL but was not phosphorylated. We generated disulfide-bonded MPL_W515L homodimers by the S402C substitution, both in the normal and KDEL context. Unlike MPL_W515L-KDEL, MPL_W515L-S402C-KDEL signaled constitutively and exhibited cell surface localization. These data establish that MPL_W515L with appended JAK2 matures through the ER/Golgi system in an inactive conformation and suggest that the MPL_W515L/JAK2 complex requires membrane localization for JAK2 phosphorylation, resulting in autonomous receptor signaling.

The thrombopoietin (TPO) receptor (MPL) signaling is critical in hematopoietic stem cell activity, megakaryopoiesis, and thrombopoiesis (1–5). MPL belongs to the homodimeric type I cytokine receptor subfamily, which includes the receptors for erythropoietin (EPO), granulocyte-colony stimulating factor, growth hormone, and prolactin (6). These receptors lack intrinsic kinase domains for the intracellular transmission of their signals (7). Instead, their signaling relies on the association with members of the cytoplasmic non-receptor Janus kinase (JAKs) family. TPO stimulation results in phosphorylation of MPL-bound JAKs and the subsequent activation of several downstream pathways, including the signal transducer and activator of transcription 5 (STAT5), Ras/mitogen-activated protein kinase, and phosphatidylinositol 3-kinase/akt (8).

Besides its function in mediating receptor signaling, JAKs also play an essential role as a chaperone protein for proper receptor cell surface localization (9). This phenomenon has been well described for the EPOR, where it was demonstrated that JAK2 binds to the receptor during its maturation processes at the endoplasmic reticulum (ER) level (10). Furthermore, it has been shown that the EPO or growth hormone receptor dimerizes in the ER in a ligand-independent fashion and before their trafficking to the cell surface (11, 12). Receptor signaling occurs at the cell surface after ligand binding and is believed to trigger changes in the dimeric receptor conformation allowing for JAK trans-phosphorylation and activation.

Recent reports have suggested that MPL homodimerization, mimicking the ligand-bound state, is sufficient for signal activation. Dimerization was induced by TPO-mimetic peptides (Fab 59, AMG 531, and peg-TPOmp), TPO nonpeptide mimetics (eltrombopag AKR-501), and TPO bivalent agonist antibodies, but also by structural modifications such as the creation of a covalent disulfide bond between two receptor chains or via the fusion of a truncated MPL with FKBPI2 and addition of FKBPI2 membrane-permeant ligand AP20187 (reviewed in Ref. 13). Based on results obtained with this latest receptor dimer, KG Otto et al. (14) have concluded that MPL signaling could occur independently of its cell surface localization. However, there is no evidence that wild type (WT) or naturally occurring active MPL mutants would adopt a dimeric conformation inside the cell.

Some primitive myelofibrosis and essential thrombocythemia patients present gain-of-function mutations in MPL targeting mainly the tryptophan at position 515 (Trp-515) that is located in the amphipathic motif at the junction between the transmembrane and intracellular domains (15–18). This motif was shown to be crucial for maintaining MPL in an inactive conformational state in the absence of ligand (19). These mutations lead to constitutive, ligand-independent activation of MPL signaling and generate a primitive myelofibrosis-like disease when expressed in murine bone marrow cells (18). The
most common mutation involves a tryptophan to leucine (W515L) substitution. They are believed to trigger conformational changes in the receptor bringing in close proximity two molecules of bound JAK2 for trans-phosphorylation and signaling. Although it is suggested that the W515L mutation leads to the formation of MPL homodimers, it is worth noting that addition of TPO to MPL (W515L)-expressing cells has an additive effect on the constitutive signaling mediated by the mutant receptor. Thus, the exact mechanisms underlying the autonomous MPL (W515L) activation in myeloproliferative disorders remain unclear.

In contrast to MPL signaling occurring at the cell surface after TPO binding, MPL (W515L) activation is not dependent on ligand binding. Therefore, it may be postulated that MPL (W515L) could signal before reaching the cell surface. This dysregulation might be involved in the pathogenesis of myeloproliferative disorders, as reported for activation of the receptor for the platelet-derived growth factor in cancer (20) or the receptor for the vascular endothelial growth factor in leukemia (21). To investigate the effect of such an abnormal intracellular signaling, we engineered a MPL (W515L) mutant molecule carrying the ER-retention KDEL sequence to prevent its cell surface expression. We expressed the KDEL-harboring or the unmodified MPL (W515L) receptor in the factor-dependent cell-progenitor 1 (FDC-P1) cell line and isolated cells expressing either the intracellular or the normally processed MPL (W515L) mutant. Our results demonstrate the strict requirement of receptor cell surface expression for MPL (W515L)-transforming activity using the FDC-P1 autonomous growth or tumor formation in nude mice as read-out. Moreover, MPL (W515L)-KDEL associated with JAK2 in the ER without activating the kinase nor the phosphorylation of STAT5, ERK1/2, and AKT signaling molecules. Interestingly, forced dimerization of MPL (W515L)-KDEL, using covalent disulfide modification, induced autonomous FDC-P1 cell growth and promoted cell surface receptor localization. These results demonstrate that MPL (W515L) binds to JAK2 in the ER/Golgi compartment in an inactive conformation and suggest that the intracellular receptor mutant is under a monomeric or a non-productive dimeric form. MPL (W515L) requires cell surface expression for ligand-independent phosphorylation and activation of JAK2.

**EXPERIMENTAL PROCEDURES**

**Cytokines and Antibodies**—Recombinant human TPO (hTPO) was from Stem Cell Technologies (Meylan, France) and murine IL-3 from PeproTech EC Ltd. (London, UK). Conditioned medium from WEHI-3B cells was used as a source of IL-3 for culture. The anti-TPO receptor/MPL monoclonal antibody (mAb) was purchased from Upstate Biotechnologies (Millipore, Saint Quentin en Yvelines, France). The anti-phospho-STAT5 Tyr-694, phospho-JAK2 Tyr-1007/1008, phospho-ERK1/2 Thr-202/Tyr-204, phospho-AKT Thr-308, and anti-JAK2 polyclonal antibodies were all from Cell Signaling Technology (Ozyme, Saint Quentin en Yvelines, France). The anti-HA (HA.11) mAb was purchased from Covance (Eurogentec, Angers, France), and mouse anti-β-actin was from Sigma (Saint Quentin Fallavier, France). mAbs were used at a concentration of 1 μg/ml and polyclonal Abs at a 1:1000 dilution for Western blotting.

**Cell Culture**—The murine FDC-P1 hematopoietic cell line is dependent for growth upon exogenous factors such as IL-3. Cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 5% WEHi-3B-conditioned medium, 2 mM l-glutamine, and antibiotics (50 IU/ml penicillin and 50 μg/ml streptomycin, Invitrogen). For retrovirus production, we used the human embryonic kidney epithelial cell line 293EBNA cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, antibiotics, and 500 μg/ml G418 (Invitrogen). Cells were grown at 90% confluence and transfected in antibiotic-free medium using Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions.

**Construction of Plasmids and Production of Retroviruses**—The cDNA encoding the human MPL (WT) was tagged with the hemagglutinin (HA) sequence cloned at the N terminus after the signal peptide cleavage site. The sequence coding for the KDEL peptide was fused with MPL C terminus. The human MPL cDNA was mutated at nucleotide 1544 (W515L) or 1206 (S402C) by the QuikChange site-directed mutagenesis method using the PfuUltra high fidelity DNA polymerase (Stratagene, Amsterdam, The Netherlands). All constructs were cloned into the bicistronic retroviral vector pMX-ires-CD4 and verified by sequencing. Viral particles pseudotyped with the vesicular stomatitis virus glycoprotein were produced into 293EBNA cells as previously described (22). FDC-P1 cells were infected with concentrated retrovirus supernatants for 2 h at a multiplicity of infection of 10 and sorted by flow cytometry (FACS Vantage, BD Biosciences, Mountain View, CA) 48 h later to isolate CD4-positive and either HA-positive or -negative cell populations.

**Expression of MPL Constructs by FACS and Western Blotting**—Surface expression of HA-MPL was analyzed by FACS after incubation of 5 × 10^5 cells in 100 μl cold phosphate-buffered saline with 10 μl of mouse anti-human CD4 mAb conjugated with allophycocyanin (APC, BD Biosciences, Le Pont de Claix, France) and mouse anti-HA mAb conjugated with R-phycocerythin (PE) (Milteni Biotec, Bergisch Gladbach, Germany) for 30 min at 4 °C. Median fluorescence intensities of PE was used for proper quantification. For Western blotting, FDC-P1 cells were directly lysed in Laemmli buffer and boiled for 10 min. Proteins extracted from 1.5 × 10^5 cells were separated by electrophoresis on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose membrane (Amersham Biosciences). Membranes were blocked with 5% bovine serum albumin (Sigma) in Tris-buffered saline-Tween buffer (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20), washed, and incubated with primary antibodies overnight at 4 °C. Horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit antibodies (Jackson ImmunoResearch, Immunotech, Marseille, France) were then added at a 1:8000 dilution for 1 h at room temperature. Labeled proteins were visualized by enhanced chemiluminescence system (Pierce, Perbio Science, Brebières, France).

**Immunoprecipitation**—Approximately 2 × 10^7 cells were harvested and lysed in 900 μl of a buffer containing 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1% Triton X-100,
500 μM NaF, 500 μM orthovanadate, and 1× protease inhibitor mixture (Roche Diagnostics, Meylan, France). For protein expression, a portion of cleared lysates was mixed with Laemmli buffer. For immunoprecipitation, 800 μl of the lysate was incubated with the anti-HA-specific mAb (20 μg/ml) overnight at 4 °C. Protein A/G PLUS-agarose (Santa Cruz Biotechnology, Tebu-Bio, Le Perray en Yvelines, France) was added to the samples, and the mixture was incubated for 1.5 h at 4 °C. The beads were washed, suspended in 45 μl of Laemmli buffer, and boiled for 10 min to release bound proteins. Samples were resolved by Western blotting.

Endo H Digestion—5 × 10^5 MPL<sub>WS15L</sub>- and MPL<sub>WS15L-KDEL</sub>-expressing FDC-P1 cells were lysed in 100 μl of the above Triton X-100-containing buffer supplemented with 0.1% SDS for 20 min on ice and digested or not with Endo H (New England Biolabs, Ozyme) for 18 h at 37 °C. The reaction was stopped by addition of Laemmli buffer and boiling for 10 min. Products were analyzed by Western blotting.

Proliferation Assay—MPL-expressing FDC-P1 cell lines were washed and cultured either in the absence of cytokine to assay for cytokine-independent growth or in presence of 10 ng/ml hTPO or 20 ng/ml mIL-3 for 7–10 days. Living cells determined by Trypan blue exclusion were manually counted at the indicated times.

Cell Tumorigenicity in Nude Mice—FDC-P1 cells (4 × 10^6 cells in phosphate-buffered saline) were injected subcutaneously into nude female mice. Five animals were used for the control (FDC-P1) and 10 for the MPL<sub>WS15L</sub> and MPL<sub>WS15L-KDEL</sub> groups. After 12 days, tumor nodules were checked, and their sizes were measured with a caliper and expressed for each group as the mean ± S.D. At days 18 and 56, tumors were dissected from 2 mice per group, and cells were mechanically dissociated with a Potter grinder for FACS analysis.

Cell Cycle Analysis—FDC-P1 cells expressing MPL<sub>WS15L</sub>, MPL<sub>WS15L-KDEL</sub>, or MPL<sub>WS15L-S402C-KDEL</sub> were washed and deprived of cytokine. After 7, 24, and 48 h in medium with no cytokine, 3 × 10^5 cells were suspended in a buffer containing 10 mM Tris-HCl, pH 7.6, 3.4 mM sodium citrate, 10 mM NaCl, 0.1% Igepal, 50 μg/ml RNase (Sigma), and 25 μg/ml propidium iodide (Sigma) and kept overnight at 4 °C. Nucleus DNA content was evaluated by FACS.

RESULTS

MPL<sub>WS15L-KDEL</sub> Is Retained Intracellularly—To evaluate whether ligand-independent activation of mutant MPL<sub>WS15L</sub> occurs intracellularly, we used the endoplasmic reticulum (ER) retention sequence KDEL to block the receptor in its process-

FIGURE 1. Cell surface and total expression of the different MPL constructs in FDC-P1 cells. A, FDC-P1 cells were transduced with the bicistronic retroviral pMX-IRES-CD4 vector encoding either the HA epitope-tagged MPL<sub>WT</sub>, MPL<sub>WS15L</sub>, or MPL<sub>WS15L-KDEL</sub>. Cells were sorted for equal CD4 levels and either HA-positive or -negative cell surface expression, accordingly, with anti-CD4-APC and anti-HA-PE antibodies. A cell population with MPL<sub>WS15L-KDEL</sub> leaking at the membrane (MPL<sub>WS15L-KDEL</sub>leak) due to the saturation of the KDEL retrieval system was selected. Sorted FDC-P1 cell populations were assessed by FACS analysis. B, median PE fluorescence intensities represent the quantities of HA-MPL localized at the cell surface, and AU represents arbitrary units. C, FDC-P1 cell lines expressing each of the MPL constructs was lysed in Laemmli buffer, and cell homogenates were resolved by SDS-PAGE. Expression of the HA-MPL constructs was examined by Western blotting with anti-HA.11 mAb (top panel). Black arrows point to three differentially glycosylated forms of MPL: 95, 80, and 65 kDa for 1, 2 and 3, respectively. The expression of β-actin serves as loading control (bottom panel). Data are representative of similar results obtained in at least three independent experiments.
ing pathway. Wild-type and mutant MPL cDNAs, modified by addition of a HA tag in the N-terminal extracellular domain, were cloned in a bicistronic retroviral vector that also expressed the human CD4 as a cell surface reporter protein. We generated vectors encoding three different MPL forms: an MPLWT mutant, a C-terminal KDEL-fused MPLMPLW515L-KDEL, and a wild-type MPLWT. Murine FDC-P1 cells were infected with each of the three retroviral supernatants. Labeling with the APC-coupled anti-CD4 antibody was used to identify virus-transduced cells and, combined with labeling with the anti-HA-PE antibody, allowed for sorting of cells expressing MPL at the cell surface (CD4-positive/HA-positive) or intracellularly (CD4-positive/HA-negative). Cells infected with the retrovirus encoding MPLMPLWT or MPLMPLW515L expressed MPL at the cell membrane (CD4+/HA+) as detected by flow cytometry (Fig. 1A). Cells infected with the retrovirus carrying MPLMPLW515L-KDEL presented two CD4-positive populations, one being HA-negative (CD4+/HA−) and one being HA-positive (CD4+/HA+). These two cell groups were independently sorted. The CD4+/HA+ cells may be the result of a defect in the KDEL retrieval system allowing the MPLMPLW515L-KDEL protein to reach or “leak” to the cell surface (Fig. 1A) and were therefore called MPLMPLW515L-KDEL leak. The CD4+/HA− cells, displaying no MPL at the cell surface were simply called MPLMPLW515L-KDEL. The median fluorescence intensities of HA-PE staining demonstrated that both MPLMPLW515L and MPLMPLW515L-KDEL leak cells expressed similar levels of MPL at the cell surface (Fig. 1B).

Total MPL proteins were examined by Western blotting with an anti-HA antibody (Fig. 1C). In general, we detected slightly less MPLWT in our cells than MPLMPLW515L. Expression of MPLMPLW515L-KDEL was clearly detected in the sorted CD4+/HA− FDC-P1 cell population (Fig. 1C), although the mutant protein was not seen at the cell surface (Fig. 1A). Physiologically, MPL undergoes glycosylation in the ER and migrates as a doublet that includes a mature endoglycosidase H (Endo H)-resistant isoform (23, 24). Indeed, MPLWT, MPLMPLW515L and MPLMPLW515L-KDEL leak cells expressed a major 80 kDa band (Fig. 1C, arrow 2) and a 95 kDa band of weak intensity, especially seen in the MPLMPLW515L-KDEL leak cells (arrow 1). The MPLMPLW515L-KDEL cells expressed two bands: the 80 kDa band and a lower size band of 65 kDa (arrow 3). The 80 and 65 kDa isoforms were sensitive to Endo H-treatment, as seen by their shift down in size, and represent maturing ER forms of MPL (Fig. 2). The 95 kDa band was resistant to Endo H and corresponds to the Golgi-processed (cell surface and intracellular mature pools) mature form of MPL. These data show that the KDEL system was efficient in retaining MPL in the ER/Golgi compartments. Furthermore, we confirm that MPL is mainly retained in the ER with only a minor fraction expressed at the cell surface.

Intracellular MPLMPLW515L Does Not Support Cytokine-independent Cell Growth—FDC-P1 cells depend upon cytokines such as IL-3 for their growth. FDC-P1 cell populations expressing the various MPL forms were tested for their ability to proliferate in absence of cytokines. As expected, all cell populations proliferated in response to IL-3 stimulation and MPLWT-expressing FDC-P1 cells grew when stimulated with TPO. Moreover, expression of MPLMPLW515L, but not MPLWT, provided autonomous cell proliferation (Fig. 3). The addition of TPO had a small but significant additive effect on the proliferation induced by MPLW515L alone. Interestingly, MPLMPLW515L-KDEL did not support factor-independent cell growth, nor did it render the cells responsive to TPO stimulation. This was not due to a defect in MPLMPLW515L function due to the fusion with the KDEL sequence on its C terminus sequence, because cells in which the
Retention system was leaking (MPL<sup>W515L</sup>-KDEL<sub>leak</sub>) grew like the MPL<sup>W515L</sup>-transduced cells in the absence of cytokines. These data demonstrate that MPL<sup>W515L</sup> needs to be expressed at the cell surface to promote cell growth.

**MPL<sup>W515L</sup> Activity Requires Cell Surface Localization**

*Intracellular MPL<sup>W515L</sup> Does Not Display Constitutive Signaling*—FDC-P1 cells expressing either MPL<sup>WT</sup> or the MPL<sup>W515L</sup> were serum- and cytokine-deprived for 4 h before being stimulated or not for 10 min with TPO (Fig. 4A). In non-stimulated condition, we detected by Western blotting a slight phosphorylation of JAK2 and of its downstream effectors STAT5, AKT and ERK solely in MPL<sup>W515L</sup>-expressing cells. These phosphorylation levels significantly increased following TPO stimulation. Similar levels of phosphorylation were observed after addition of TPO to cells expressing MPL<sup>WT</sup>. This data are in agreement with the proliferation assay (Fig. 3).

Similar results were observed for MPL<sup>W515L</sup>-KDEL expressing cells. In contrast to MPL<sup>W515L</sup>, MPL<sup>W515L</sup>-KDEL did not activate any of its signalization partners in the absence of stimulation or in the presence of TPO. The addition of IL-3 induced similar phosphorylation levels of JAK2 and its effectors in the three cell lines (Fig. 4B). These results show that MPL<sup>W515L</sup> activity requests cell surface localization.

*Intracellular MPL<sup>W515L</sup> Is Not Tumorigenic in Mice*—To assay the tumorigenic potential of MPL<sup>W515L</sup> and MPL<sup>W515L</sup>-KDEL, FDC-P1 cells expressing each receptor were subcutaneously injected into nude mice. Throughout the experiment, none of the mice injected with parental FDC-P1 cells developed any tumor. In contrast, 1 week after injection, 70% of the mice injected with MPL<sup>W515L</sup>-expressing FDC-P1 cells developed tumors of significant size. Surprisingly, small tumor nodules were seen in 20% of the mice injected with MPL<sup>W515L</sup>-.
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KDEL-expressing cells (Fig. 5A). At day 56, oversized tumors were seen in all engrafted animals. Tumors were dissected from two mice of each group at days 18 and 56 (Fig. 5B). Cells were dissociated and labeled with anti-CD4-APC and anti-HA-PE antibodies for FACS analysis. CD4-positive cells recovered from tumors generated after injection of MPL<sub>W515L</sub>-expressing cells showed a significant receptor surface expression (HA labeling) at days 0, 18 and 56. As previously seen, HA-labeling was inexistent in MPL<sub>W515L</sub>-KDEL cells at day 0. However, cell surface expression of MPL<sub>W515L-KDEL</sub> became detectable at a low level at day 18 and reached a level similar to MPL<sub>W515L</sub> in cells isolated from oversized tumors at day 56. These data strongly suggest that MPL<sub>W515L</sub> must be expressed at the cell surface for its tumorigenic activity.

**JAK2 Binds Intracellularly to MPL<sub>W515L</sub> but Without Being Phosphorylated**—Next, we investigated whether the absence of transforming activity of MPL<sub>W515L-KDEL</sub> could be attributed to an absence of JAK2 binding to MPL in the ER. We first verified whether and to what extent MPL<sub>WT</sub> could bind to JAK2. MPL<sub>WT</sub>- and MPL<sub>W515L</sub>-expressing cells were serum- and cytokine-deprived for 4 h before stimulation or not with TPO (Fig. 6A). MPL was immunoprecipitated using the HA tag. In basal and stimulated conditions, JAK2 was associated to both MPL<sub>WT</sub> and MPL<sub>W515L</sub> (Fig. 6A, second panel from top). In MPL<sub>W515L</sub>-expressing cells only we detected a weak phosphorylation of bound JAK2 in non-stimulated conditions (Fig. 6, A and B, top panels). As expected, addition of TPO resulted in an increase in the phosphorylation of both MPL<sub>WT</sub>- and, to a greater extent, MPL<sub>W515L</sub>-bound JAK2. Similarly, MPL<sub>W515L</sub>- and MPL<sub>W515L-KDEL</sub>-expressing cells were stimulated or not with TPO or IL-3 before immunoprecipitating the HA tag of MPL. Interestingly, results showed that JAK2 was bound to both MPL<sub>W515L</sub> and MPL<sub>W515L-KDEL</sub> at a similar extent in basal and stimulated conditions (Fig. 6B, second panel from top). Then, we verified the phosphorylation status of the MPL-bound JAK2. Phospho-JAK2 bound to MPL<sub>W515L</sub> was clearly detected in TPO-stimulated cells and to a lesser extent in non-stimulated or IL-3-stimulated cells (Fig. 6B, top panel). In contrast, JAK2 bound to MPL<sub>W515L-KDEL</sub> was not phosphorylated under any condition. These data indicate that JAK2 and MPL associate intracellularly during the ER/Golgi maturation steps and that cell surface localization acquisition is necessary for phosphorylation of JAK2 associated to the MPL<sub>W515L</sub> mutant receptor.

**Forced Dimerization of the MPL<sub>W515L</sub>-KDEL Induced Factor-independent Cell Growth but Also Receptor Cell Surface Expression**—Whether MPL homodimerizes in the ER is unknown. It is possible that MPL monomers bind to JAK2 in the ER and that the complex reaches the cell surface before W515L mutation induces dimerization leading to JAK2 auto-phosphorylation. However, an inactive homodimeric form of the receptor may be generated intracellularly as well. To investigate the role of dimerization in MPL<sub>W515L</sub> activation, we developed a preformed MPL<sub>W515L</sub> dimer that could be compared with MPL<sub>W515L</sub>. As previously obtained with mouse MPL, dimerization was initiated by substituting a Ser with a Cys at position 402 in the membrane-proximal extracellular domain (equivalent of mouse S368C) to form the creation of a covalent disulfide bond. This modification was shown to induce constitutive activity of mouse MPL (25). We first verified that it was also the case for human MPL<sub>WT</sub>-S402C. Firstly, treatment of lysates of cells expressing MPL<sub>WT</sub>-S402C with β-mercaptoethanol reduced species from a dimeric to a monomeric size on a Western blot probing with the anti-HA mAb, indicating the formation of 20.3% (Image) software, NIH, USA) of MPL<sub>WT</sub> dimers as a result of the S402C mutation (Fig. 7A). Secondly, we observed no major difference in cell surface expression of MPL<sub>WT</sub>-S402C compare with MPL<sub>WT</sub> by FACS analysis (Fig. 7B). Finally, the human MPL<sub>WT</sub>-S402C mutant was found to allow autonomous cell growth (Fig. 7C).

Similarly, the S402C substitution induced dimerization of 4.1% of MPL<sub>W515L</sub> and 7.9% MPL<sub>W515L-KDEL</sub> (Fig. 8A). MPL<sub>W515L-KDEL</sub>- and MPL<sub>W515L-S402C-KDEL</sub>-expressing cells were sorted for retention of the receptors in the ER, as
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shown by FACS analysis (Fig. 8B), and tested for autonomous growth. Surprisingly, while MPL<sub>W515L</sub>-KDEL-expressing cells died within 3 days in the absence of cytokine, MPL<sub>W515L-S402C</sub>-KDEL-expressing cells grew in the same condition (Fig. 8C). However, FACS analysis revealed that the factor-independent MPL<sub>W515L-S402C</sub>-KDEL-expressing cells expressed the mutant receptor at the cell surface at low level at day 3 but significant level at day 7 (Fig. 8D). The cumulative cell numbers for MPL<sub>W515L-S402C</sub>-KDEL, in the absence of cytokine, were plotted on a linear scale between day 0 and day 3 (Fig. 8E) and demonstrated active cell proliferation rather than solely cell survival. These data show that the S402C dimer form of MPL<sub>W515L</sub> can signal in the absence of TPO but also overrides the intracellular retention induced by the KDEL signal. Thus, forced dimerization promotes both intracellular signaling of MPL<sub>W515L</sub> and cell surface localization of an internally locked MPL<sub>W515L</sub>. We further verified that MPL<sub>W515L-S402C</sub>-KDEL-expressing FDC-P1 cells were actively dividing in non-stimulated conditions during the first 3 days, thus before cell surface receptor leakage. Cell cycle assay based on iodure propidium staining of nuclei established that MPL<sub>W515L</sub>-KDEL-expressing cells arrested in G<sub>1</sub> phase and entered in apoptosis, as seen with the appearance of a SubG<sub>1</sub> phase (Fig. 8F), as early as 24 h after cytokine deprivation. In contrast, both MPL<sub>W515L</sub>- and MPL<sub>W515L-S402C</sub>-KDEL-expressing cells were driven into S phase as early as 7, 24, and 48 h after cytokine-deprivation (Fig. 8F).

Cells expressing the diverse forms of MPL were serum- and cytokine-starved for 4 h before 10-min stimulation or not with TPO. Level of JAK2 phosphorylation was studied by Western blotting (Fig. 9A). As expected, JAK2 phosphorylation strongly increased in MPL<sub>WT</sub>- and MPL<sub>W515L</sub>-expressing cells after TPO addition. However, the forced dimerized MPL<sub>W515L-S402C</sub> or MPL<sub>W515L-S402C</sub>-KDEL mutant, in contrast to MPL<sub>WT</sub>- and MPL<sub>W515L</sub>, seemed to be unresponsive to further TPO stimulation. This result suggests that dimerization achieves full endogenous activation of MPL<sub>W515L</sub>, but not of MPL<sub>WT</sub> that still needs TPO to reach full activation. Finally, JAK2 co-immunoprecipitated with both MPL<sub>W515L</sub> and MPL<sub>W515L-S402C</sub> in a TPO-independent fashion (Fig. 9B, second panel from top). As expected from the previous result, TPO stimulation resulted in an increased phosphorylation of JAK2 bound to MPL<sub>W515L</sub> but had no significant effect on the basal level of phospho-JAK2 associated with the MPL<sub>W515L-S402C</sub> (top panel). Taken together, these data suggest that MPL<sub>W515L</sub> carrying the S402C mutation further rearranges in a conformation similar to the ligand-bound MPL. Our results strongly imply that MPL<sub>W515L</sub>, associated with JAK2, is not active before arriving at the cell surface and that dimerization is necessary for its full activation.

**DISCUSSION**

Oncogenic mutations in the TPO receptor, MPL, have been uncovered in a minority of primitive myelofibrosis and essential thrombocythemia (18). In particular, a cryptophan substitution at position 515 by a leucine (W515L) was described within an amphipathic RWQPF motif at the juxtamembrane region (19). Such a substitution leads to constitutive MPL signaling (15–18). This discovery raised questions on the molecular mechanisms at the basis of MPL mutant transforming activity. A major finding of this study is the demonstration that MPL<sub>W515L</sub> cannot signal during its intracellular processing pathway, but requires cell surface localization. In addition, our data show that MPL associates with JAK2 in the ER in an inactive conformation that is either a monomer or a non-productive dimer. Ligand-independent homodimerization in a productive conformation for signaling may occurs when MPL<sub>W515L</sub> reaches the plasma membrane.

As a member of the homodimeric type I cytokine receptor subfamily, MPL is often compared with its counterpart, the erythropoietin receptor. EPOR has been extensively studied. It is well demonstrated that EPOR undergoes dimerization in the absence of ligand (26) and associates with its effector kinase JAK2 during maturation in the ER (10, 12). Indeed, JAK2 has been shown to play an essential role, as a chaperone protein, for proper maturation and cell surface expression, not only for
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A.

![Western Blot Image]

B.

![Graph Image]

C.

![Cell Counts Graphs]

D.

![Bar Graph]

E.

![Graph Image]

F.

![Flow Cytometry Graphs]
MPL<sup>W515L</sup> Activity Requires Cell Surface Localization

A population of FDC-P1 cells where MPL<sup>W515L</sup>-KDEL was efficiently retained intracellularly. ER localization was suggested by a preponderant perinuclear immunofluorescent staining (data not shown) and by Western blotting analysis. The MPL<sup>W515L</sup> was revealed as a doublet with the major band corresponding to the maturing 80-kDa form sensitive to Endo H. These data are reminiscent of the finding that only extremely low levels of EPOR are expressed at the cell surface (28). Notably, MPL<sup>W515L</sup>-KDEL presented as two Endo H-sensitive bands. The preponderant form was a 65-kDa band likely corresponding to the ER-retained form. A minority of the KDEL mutant receptor also migrated as the 80-kDa band detected for MPL<sup>W515L</sup>. Thus, this suggests that MPL<sup>W515L</sup>-KDEL is formed in the majority of the ER but also throughout the Golgi stack. Indeed, the capacity to retrieve escaped KDEL-tagged proteins extends to the trans-Golgi network although, in theory, receptors recognizing the KDEL sequence are denser in the ER and cis-Golgi (29). Therefore, this system allowed us to look for the potential of MPL<sup>W515L</sup> to signal not only at the ER level but throughout its maturation pathway at the exclusion of the mature cell surface form.

When retained inside the cells, MPL<sup>W515L</sup>-KDEL was unable to support the autonomous growth of the factor-dependent FDC-P1 cell line. This was confirmed by the absence of signal such as phosphorylation of STAT5, AKT, and ERK1/2. Moreover, the MPL<sup>W515L</sup>-KDEL-expressing cells became tumorigenic in nude mice only after a clear selection of clones with escaped receptors at the cell surface had occurred in vivo. The lack of biological activity of MPL<sup>W515L</sup>-KDEL raised one major concern, i.e., the possibility that addition of the KDEL sequence at the C terminus of MPL would modify the structure of the receptor. When FDC-P1 cells infected with the MPL<sup>W515L</sup>-KDEL-expressing retrovirus were sorted, we selected an HA-negative population (no MPL surface expression) and a HA-positive cell fraction (MPL surface expression) that we called MPL<sup>W515L</sup>-KDEL<sup>leak</sup>. These cells expressed the KDEL-modified MPL<sup>W515L</sup> at the cell surface likely, because saturation of the FDC-P1 KDEL retrieval system occurs. "Leaking" cells may have a saturated or defective ER retention system with for instance higher amounts of endogenous KDEL proteins such as ER-resident chaperones, thus competing with MPL<sup>W515L</sup>-KDEL for binding to the KDEL receptors, or less KDEL receptors, respectively. Nevertheless, when expressed at the cell surface, MPL<sup>W515L</sup>-KDEL<sup>leak</sup> sustained the factor-independent proliferation of FDC-P1 and displayed a TPO-dependent activity like MPL<sup>W515L</sup> demonstrating that fusion with the KDEL sequence did not impaired receptor function.

EPOR but also for MPL (9, 10). At the cell surface, ligand binding induces conformational changes in the receptor complex leading to the trans-activation of the associated JAK2 (27).

We explored the theory that MPL<sup>W515L</sup> signaling could occur before the receptor reached the cell surface and induce a pathological signal as demonstrated in cancer with other receptors (20, 21). We used the ER retention KDEL sequence to block MPL<sup>W515L</sup> within its natural processing pathway. Indeed, MPL via its peptide signal is targeted to the ER/Golgi complex where it undergoes folding, N-glycosylation, and correct maturation before being expressed at the cell membrane (24). We selected...
An important concept in receptor signaling is the spatial and temporal co-localization with its partners (30). The subcellular localization of MPL to specific microdomains remains unexplored. In particular, the cholesterol-glycosphingolipid-rich domains, also called lipid rafts, are enriched in JAK2, ERK1/2, and AKT and may be an environment of choice for MPL to mediate signaling (31). Whereas certain STATs have been identified in these plasma membrane invaginations, the presence of STAT5 is more questionable. The lack of signaling might be due in part to differences in protein contents between the ER and the cell surface environments. Yet, co-immunoprecipitation studies showed that MPL\textsuperscript{W515L}, KDEL associates with its main signaling kinase, JAK2.

The co-immunoprecipitation of JAK2 with MPL\textsuperscript{W515L-KDEL} implies that JAK2 associates with MPL\textsuperscript{W515L} at the level of the ER/Golgi as previously demonstrated for EPOR (10). This result indicates that the absence of intracellular MPL\textsuperscript{W515L} activity was not related to the absence of JAK2 binding. Thus, we investigated whether the lack of intracellular MPL\textsuperscript{W515L} signaling was due to the absence of productive intracellular dimerization, possibly due to its immature glycosylation status. Cysteine substitution in an extracellular region of growth hormone receptor EPOR, and MPL, designed to promote disulfide-bonded homodimerization, has allowed for the identification of a conserved dimer interface domain between these receptors (32, 33). In particular, the S368C substitution in the murine receptor (25). Using this last strategy, we generated a dimerized MPL was demonstrated to induce constitutive activation of the main signaling kinase, JAK2.

This result indicates that the absence of intracellular MPL\textsuperscript{W515L} activity was not related to the absence of MPL\textsuperscript{W515L}-JAK2. Combined with the observations that the fraction of human MPL (S402C) mutant and found that MPL\textsuperscript{WT-S402C}, like MPL\textsuperscript{W515L-S402C}, displayed a TPO-independent activity on cell growth and constitutive JAK2 phosphorylation. Interestingly, the presence of TPO had no additive effect on JAK2 activation by the double mutant MPL\textsuperscript{W515L-S402C}, in contrast to MPL\textsuperscript{W515L}. This result shows that dimerization leads to full endogenous activity of MPL\textsuperscript{W515L}, possibly by mimicking the conformation of ligand binding on MPL\textsuperscript{W515L}.

Strikingly, FDC-P1 cells expressing MPL\textsuperscript{W515L-S402C-KDEL} acquired the ability to grow in absence of cytokines. In a cytokine-deprived medium, MPL\textsuperscript{W515L-KDEL} was retained in the ER and cells entered apoptosis within a few hours. During 2–3 days in similar conditions, the dimeric MPL\textsuperscript{W515L-S402C-KDEL} was also retained in the ER but sustained autonomous cell growth. Thereafter, the KDEL retention system was apparently overridden, because MPL appeared at the cell surface. Although we cannot distinguish between a very low cell surface localization leading to weak signaling and intracellular signaling for the MPL\textsuperscript{W515L-S402C-KDEL} mutant, these data suggest that dimerization plays a role similar to cell surface processing in the activation of MPL\textsuperscript{W515L} signaling.

The activating W515L mutation is found in a region thought to prevent two MPL strands from assuming an active dimeric conformation, when the ligand is not bound (19). Cycloheximide treatment indicates that locating in the ER seemed to substantially prolong the half-life of MPL\textsuperscript{W515L} (data not shown). Combined with the observations that the fraction of surface MPL is very low and that JAK2 bound to MPL\textsuperscript{W515L-KDEL} is not phosphorylated, our data suggest that MPL\textsuperscript{W515L} may be processed through the ER and Golgi systems as a single strand, associated with JAK2, or as an inactive dimer, and would assume an active homodimeric conformation when reaching the cell surface. Only at the cell surface would the bound JAK2 be phosphorylated. Ligand binding would trigger an additional activating conformational rearrangement in MPL\textsuperscript{W515L}. On a clinical side, it should be mentioned that MPL protein expression is decreased in PV and primitive myelofibrosis patients, and that glycosylation and surface expression are impaired (23, 34). Thus, it would be interesting to understand whether this phenotype is linked to the MPL status (WT or W515L).

To our knowledge, this is the first report suggesting that MPL\textsuperscript{W515L} may mature through the ER and Golgi apparatus in an inactive conformation bound to JAK2 before reaching the cell surface where it would acquire an active homodimeric conformation. This processing would be applicable to the wild-type MPL and might be a way to control signal dysregulation by keeping the JAK2 molecules apart and therefore inactive before trafficking to the cell surface is achieved.

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REFERENCES
1. Broudy, V. C., and Kaushansky, K. (1995) J. Leukocyte Biol. 57, 719–725
2. Gurney, A. L., Carver-Moore, K., de Sauvage, F. J., and Moore, M. W. (1994) Science 265, 1445–1447
3. Kaushansky, K., Sok, L., Holly, R. D., Broudy, V. C., Lin, N., Bailey, M. C., Forstrom, J. W., Buddle, M. M., Oort, P. J., Hagen, F. S., Roth, G. J., Papa-yannopoulois, T., and Foster, D. C. (1994) Nature 368, 568–571
4. Vainchenker, W., Methia, N., Debili, N., Titeux, M., and Wendling, F. (1995) Thromb. Haemostasis 74, 526–528
5. Wendling, F. (1999) Haematologica 84, 158–166
6. Cosman, D., Lyman, S. D., Iderza, R. L., Beckmann, M. P., Park, L. S., Goodwin, R. G., and March, C. J. (1990) Trends Biochem. Sci. 15, 265–270
7. Parganas, E., Wang, D., Stravopodis, D., Topham, D. J., Marine, J. C., Teglund, S., Vanin, E. F., Bodner, S., Colamonici, O. R., van Deursen, J. M., Grosveld, G., and Iheu, J. N. (1998) Cell 93, 385–395
8. Yamaoka, K., Saharinen, P., Pesu, M., Holt, V. E., 3rd, Silvennoinen, O., and O'Shea, J. J. (2004) Genome Biol. 5, 253
9. Royer, Y., Staerk, J., Costuleanu, M., Courtoy, P. J., and Constantinescu, S. N. (2005) J. Biol. Chem. 280, 27251–27261
10. Huang, L. J., Constantinescu, S. N., and Lodish, H. F. (2001) Mol. Cell 8, 1327–1338
11. Gent, I., van Kerkhof, P., Roza, M., Bu, G., and Strous, G. J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 9858–9863
12. Livnah, O., Stura, E. A., Middleton, S. A., Johnson, D. L., Jolliffe, L. K., and Wilson, I. A. (1999) Science 283, 987–990
13. Kuter, D. J. (2007) Blood 109, 4607–4616
14. Otto, K. G., Broudy, V. C., Lin, N. L., Parganas, E., Luthi, J. N., Drachman, J. G., Iheu, J. N., and Blau, C. A. (2001) Blood 98, 2077–2083
15. Beer, P. A., Campbell, P. J., Scott, L. M., Bench, A. J., Erber, W. N., Bareford, D., Wilkins, B. S., Reilly, J. T., Hasselbach, H. C., Bowman, R., Wheatley, K., Back, G., Harrison, C. N., and Green, A. R. (2008) Blood 112, 141–149
16. Chaligine, R., James, C., Tometti, C., Besancenot, R., Le Couedic, J. P., Parganas, E., Luthi, J. N., Drachman, J. G., Iheu, J. N., and Blau, C. A. (2001) Blood 98, 2077–2083
17. Chaligine, R., James, C., Tometti, C., Besancenot, R., Le Couedic, J. P., Fava, F., Mazurier, F., Godin, I., & Litzow, M. R., Gilliland, D. G., and Tefferi, A. (2006) Blood 108, 3735–3743
18. Pardanani, A. D., Levine, R. L., Lasho, T., Poksik, Y., Masa, R. A., Wadleigh, M., Steensma, D. P., Elliott, M. A., Wolansky, A. P., Ebert, W. J., McClure, R. F., Litzow, M. R., Gilliland, D. G., and Tefferi, A. (2006) Blood 108, 3735–3743
19. Pikman, Y., Lee, B. H., Mercher, T., McDowell, E., Ebert, B. L., Gozo, M., Cuker, A., Wernig, G., Moore, S., Galinsky, I., DeAngelo, D. J., Clark, J. I., Lee, S. J., Golub, T. R., Wadleigh, M., Gilliland, D. G., and Levine, R. L. (2006) Blood 108, 3735–3743
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26. Constantinescu, S. N., Keren, T., Socolovsky, M., Nam, H., Henis, Y. I., and Lodish, H. F. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 4379–4384
27. Constantinescu, S. N., Huang, L. J., Nam, H., and Lodish, H. F. (2001) Mol. Cell 7, 377–385
28. D’Andrea, A. D., and Zon, L. I. (1990) J. Clin. Investig. 86, 681–687
29. Miesenbock, G., and Rothman, J. E. (1995) J. Cell Biol. 129, 309–319
30. Jacobson, K., Sheets, E. D., and Simson, R. (1995) Science 268, 1441–1442
31. Brown, D. A., and London, E. (2000) J. Biol. Chem. 275, 17221–17224
32. Watowich, S. S., Yoshimura, A., Longmore, G. D., Hilton, D. J., Yoshimura, Y., and Lodish, H. F. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 2140–2144
33. Yoshimura, A., Longmore, G., and Lodish, H. F. (1990) Nature 348, 647–649
34. Tefferi, A., Yoon, S. Y., and Li, C. Y. (2000) Blood 96, 771–772