Brief report

The Asn505 mutation of the c-MPL gene, which causes familial essential thrombocythemia, induces autonomous homodimerization of the c-Mpl protein due to strong amino acid polarity

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We previously reported that a dominant-positive activating mutation (Asn505) in the transmembrane domain (TMD) of c-MPL, which encodes the thrombopoietin receptor, caused familial essential thrombocythemia. Here, we show that the Asn505 mutation induces both autonomous dimerization of c-Mpl and signal activation in the absence of its ligand.

Signal activation was preserved in a truncated mutant of Asn505 that lacked the extracellular domain of c-MPL. We also found that the substitution of the amino acid (AA) residue at position 505 with others of strong polarity (Glu, Asp, or Gin) also resulted in activated dimerization without ligand stimulation. Overall, these data show that the Asn505 mutation transduced the signal through the autonomous dimerization of the c-MPL protein due to strong AA polarity. This finding provides a new insight into the mechanism of disease causation by mutations in the TMD of cytokine/hematopoietic receptors.

Methods

Wild-type and mutant c-MPL were inserted into pCI-Neo vectors14 and used as templates for PCR amplification. The primers 5′-tactacgcgttgagc-3′ and 3′-tatgaggactgctgctgact-5′ were designed to amplify the truncated mutants ΔSer505 and ΔAsn505 that lack the entire ECD, corresponding to AA 490-636 of c-Mpl. The products were inserted into pCI-Neo with MilI and NotI sites.

We also generated a range of c-MPL mutants at position 505 instead of wild-type Ser using the Quick-Change Multi Site-Directed Mutagenesis kit (Stratagen). The constructs were stably transfected into Ba/F3 cells, and their sequences and protein expressions were examined by direct sequencing and immunoblotting, respectively.14 The membrane localization of their c-MPL proteins were confirmed (Figure 1A-B).

Cytokine/hematopoietic receptors possess a single transmembrane domain (TMD). Upon stimulation by ligand binding, some receptors transduce signals into the cytoplasm through dimerization and conformational changes in preformed dimers, and thereby activate a cascade of transphosphorylation signals, such as Jak2, Mek1/2, and Stat5.1-8 The chemical properties of amino acid (AA) residues in the TMD are thought to play important roles in receptor dimerization and signal activation.8,9 Activating mutations in the TMD of cytokine/hematopoietic receptors have been identified because of oncogenic activity or as a cause of human diseases. For example, Val664Glu in neu/ErbB2-R (Blood. 2009;114:3325-3328) acts as an oncogene, while Gly380Arg and Ala391Glu in fibroblast growth factor receptor 3 (FGFR3) cause achondroplasia and Crouzon syndrome with acanthosis nigricans.11,12

Onishi et al identified an activating mutation in the TMD of c-MPL using polymerase chain reaction (PCR)–driven random mutagenesis13; this mutation (Asn505) was demonstrated to be a cause of familial essential thrombocythemia (FET).14 However, it was unclear how Asn505 constitutively activated intracellular signaling. Here, we investigated autonomous dimerization in Asn505 and in a truncated mutant of the entire extracellular domain (ECD) of c-MPL. We also tested whether the substitution of AA residues at the mutated position 505 of c-MPL influenced dimerization and intracellular signaling.

Results and discussion

Homodimerization and conformational changes in receptors have been shown to be essential for signal activation in some cytokine/
hematopoietic receptors. Thus, we evaluated homodimerization in the various c-MPL–expressing Ba/F3 cells under nonreducing conditions with the chemical cross-linker BS1. In Tpo-free conditions, Asn505-expressing cells, but not Ser505-expressing cells, contained a band double the size of the c-MPL monomer (Figure 1A). The detection of dimerized bands depended on the presence of BS1, suggesting that dimerization was not due to a disulfide bond. TPO-independent phosphorylation of MEK1/2 and STAT5 was detected by the occurrence of the dimerized bands.

Previous studies showed that the ECD of cytokine receptors mediated dimerization and controlled receptor activity. Thus, we generated 2 c-MPL mutants, ∆Ser505 and ∆Asn505, in which the entire ECD was deleted. The ∆Asn505 mutant displayed homodimerization with the membrane-permeable cross-linker DSG and phosphorylation of MEK1/2 and STAT5 independently of IL-3 stimulation (Figure 1B). Cells carrying ∆Asn505 survived (but did not proliferate) in factor-free conditions, similarly to the Asn505 mutant (Figure 1C). These data indicate that the Asn505 mutation induced autonomous homodimerization and intracellular activation independently of growth factors and the ECD of c-MPL (Figure 1D).

The functions of the cytoplasmic domain and the ECD of c-MPL have been described in various reports. Our observation that ∆Ser505 did not induce constitutive activation (Figure 1B) is of interest because Sabath et al previously showed that deletion of the distal ECD (CRM-1) induced
Constitutive activation independently of TPO stimulation. We also found that ΔAsn505 had weaker phosphorylation of intracellular signals (Figure 1B), suggesting that the ECD of c-MPL may contribute to the stabilization of dimerization and signaling. The precise function of the ECD of c-MPL will be confirmed with further analysis using such mutants.

In the Asn505 mutant, MEK1/2 and STAT5 were phosphorylated more weakly without TPO stimulation than with TPO (Figure 1A), and cell survival rather than proliferation was observed without TPO. Under TPO stimulation, the proliferation capacity of the Asn505 mutant was not significantly different from the wild-type (Ser505; data not shown); this is consistent with another study. These findings suggest that Asn505 is not a fully constitutive activating mutation and does not have hypersensitivity to TPO; in the absence of factor stimulation. Cells transfected with mutants with 2-polar residues (Glu, Asp, or Gln) showed factor-independent cell survival. Mock and Ba/F3 cells were transfected with PCI-Neo vector. The results are shown as means ± SEM of 3 separate experiments. (C) Autonomous homodimerization and signal activation of the artificial TMD mutants of c-MPL. (A) A list of the AA substitution mutants generated at position Ser505 of c-MPL. All mutants were transfected into Ba/F3 cells. (B) MTT assay of the artificial TMD mutants of c-MPL. The top panel shows the detection of homodimers in immunoblots under nonreducing conditions. BS3 was used as the cross-linker.

The Asn residue in the TMD of receptors was previously shown to induce interhelical hydrogen bonds with strong polarity. Thus, we hypothesized that autonomous dimerization in Asn505 was induced by hydrogen bonding due to its strong polarity. To test this, we examined dimerization in various Ba/F3/c-Mpl mutants in which Ser505 was substituted with residues with different polarities: 2-polar Glu, Asp, or Gln; 1-polar Lys or Thr; and nonpolar Val or Leu (Figure 2A). The mutants with 2-polar residues presented factor-independent cell survival and constitutive phosphorylation of MEK1/2 and STAT5 with dimer formation, but the mutants with a 1-polar or nonpolar residue did not show such effects (Figure 2B-C). These results strongly suggest that autonomous dimerization of c-MPL in Asn505 was induced by hydrogen bonding due to strong AA polarity.

We reviewed all the reported activating mutations in the TMD of cytokine receptors (supplemental Table 1, available on the Blood website; see the Supplemental Materials link at the top of the online article) and found that most contain AA substitutions that result in selective delay in the down-regulation of the mutant receptor,11 these mutations appear to have a similar effect to Asn505 in c-MPL. The exceptions were the mutations of a 1-polar Arg residue in FGFR223 and FGFR3,11 Arg380 of FGFR3 predominantly showed ligand-dependent dimerization and overexpression with a selective delay in the down-regulation of the mutant receptor,11 suggesting a different mechanism from Asn505. Our future studies will include 3-D structural analyses,24,25 which should be valuable for understanding the precise mechanism by which Asn505 exerts its effects.

Overall, the Asn505 mutation of c-MPL, a cause of FET, activates intracellular signals through homodimerization in a
factor-independent manner. Homodimerization resulted from hydrogen bonding due to the strong polarity of the Asn residue. Our results have provided a new insight into the mechanism of activating mutations in the TMD of cytokine/hematopoietic receptors.

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References


Authorship

Contribution: J.D. designed and performed the experiment and wrote the paper; H.K., S.I., and M.N. designed research and discussed the data; H.Y., A. Inagaki, F.M., and A. Ito contributed to the design of the research and discussed the data; and R.U. guided the research.

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