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M-CSF receptor mutations in hereditary diffuse leukoencephalopathy with spheroids impair not only kinase activity but also surface expression

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ABSTRACT

The tyrosine kinase Fms, the cell surface receptor for M-CSF and IL-34, is critical for microglial proliferation and differentiation in the brain. Recently, a number of mutations have been identified in Fms as a putative genetic cause of hereditary diffuse leukoencephalopathy with spheroids (HDLS), implying an important role of microglial dysfunction in HDLS pathogenesis. In this study, we initially confirmed that 11 mutations, which reside within the ATP-binding or major tyrosine kinase domain, caused a severe impairment of ligand-induced Fms auto-phosphorylation. Intriguingly, we found that 10 of the 11 mutants also showed a weak cell surface expression, which was associated with a concomitant increase in the low molecular weight hypo-*N*-glycosylated immature gp130Fms-like species. Indeed, the mutant proteins heavily accumulated to the Golgi-like perinuclear regions. These results indicate that all of the Fms mutations tested severely impair the kinase activity and most of the mutations also impair the trafficking to the cell surface, further suggesting that HDLS is caused by the loss of Fms function.

1. Introduction

The differentiation, survival, and function of cells of the mononuclear phagocyte lineage are regulated by the tyrosine kinase Fms [1,2]. The binding of Fms to its ligand M-CSF (also known as CSF-1) at the cell surface induces the dimerization of Fms and the activation of its tyrosine kinase activity, resulting in the autophosphorylation of multiple tyrosine residues [1,2]. IL-34 is another functional ligand of Fms [3]. The alternative ligand explains the fact that most phenotypic defects in M-CSF-deficient *op*/*op* mice are more severe in Fms-deficient mice [4]. Indeed, studies with IL-34-deficient mice demonstrated that IL-34, which is highly expressed in neurons, but not M-CSF, is required for the development of microglia in the brain [5,6]. Moreover, we demonstrated that M-CSF and IL-34, which share little sequence homology [3], bind to different domains of Fms, and thereby induce different bioactivities and signaling activation kinetics/strength [7].

Importantly, Rademakers et al. recently identified 14 different mutations in Fms, including missense, frame-shift and splice-site mutations, in patients with hereditary diffuse leukoencephalopathy with spheroids (HDLS), an autosomal-dominant central nervous system white matter disease [8]. Subsequent studies also identified Fms mutations in a significant proportion of clinically and pathologically proven HDLS [9–17]. These mutations, which reside within the tyrosine kinase domain, are considered to affect the catalytic activity of Fms and presumably microglial dysfunction [8]. Indeed, 3 mutants ($E^{633}K$, $M^{766}T$, and $M^{875}T$) showed a severely impaired M-CSF-induced auto-phosphorylation [8]. However, it is possible that some of the remaining mutants allow residual kinase activity. Moreover, as suggested in the previous study [8], it is possible that these mutations affect the ligand-induced dimerization or cell surface expression itself. In this study, we therefore examined these possibilities in 11 different mutants.

2. Materials and methods

2.1. Fms mutant plasmids

We prepared 11 different HDLS-related Fms mutants [8] using the QuikChange II site-directed mutagenesis kit (Stratagene), appropriate primers, and the wild-type human Fms [18] cloned into pIRESneo3 vector (Clontech) as a template. Their nucleotide sequences were verified using BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) and an ABI PRISM 3100 genetic analyzer (Applied Biosystems).

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2.2. Cytokines and cells

Recombinant human (rh) M-CSF was a gift from Morinaga Milk Industry (Kanagawa, Japan). rhIL-34 was purchased from R&D Systems. The 293A cells (Invitrogen) were transfected with plasmid for the wild-type or mutant Fms using Lipofectamine 2000 reagent (Invitrogen) and then selected in DMEM-10% FCS containing 1.2 mg/ml G418 (Calbiochem) to establish bulk cells stably expressing these Fms proteins.

2.3. Western blotting

The 293 cells stably expressing Fms proteins were stimulated with M-CSF or IL-34, and lysed with Nonidet P-40 buffer containing protease inhibitors and phosphatase inhibitors, as described previously [7]. Total cell lysates were then subjected to Western blotting. The following antibodies were used: anti-Fms (C-20; Santa Cruz Biotechnology), anti-phospho-specific Fms (pY^{546}, pY^{699}, pY^{708}, pY^{723}, pY^{809}, and pY^{923}; all from Cell Signaling), and anti-actin (C-2; Santa Cruz Biotechnology). Detection was performed with HRP-labeled secondary antibodies (GE Healthcare), Immunostar LD Western blotting detection reagent (Wako, Osaka, Japan), and an image analyzer (ImageQuant LAS 4000; GE Healthcare).

2.4. Flow cytometry

The cell surface expression of Fms was assessed by flow cytometry on a guava easyCyte HT flow cytometer (Millipore). The cells were detached from the culture dishes using enzyme-free cell dissociation buffer (Gibco), and stained with PE-labeled anti-Fms antibodies (3-4A4-E4; Santa Cruz Biotechnology), as described previously [7]. The binding of Flag-tagged M-CSF to the surface of cells expressing Fms was also assessed by flow cytometry-based analysis [7]. The cells were incubated sequentially with Flag-tagged M-CSF proteins, biotin-labeled anti-Flag antibodies (M2; Sigma), and PE-labeled streptavidin (PharMingen).

2.5. Immunostaining

Immunostaining was performed as described previously [18]. The cells cultured on slides coated with fibronectin (Roche) were fixed in 2% paraformaldehyde, permeabilized with PBS containing 0.2% Triton X-100, and stained with anti-Fms (C-20; Santa Cruz, Santa Cruz, CA), anti-GM130 (clone 35; BD Transduction) or anti-EEA-1 antibodies (clone 14; BD Transduction) followed by fluorescent dye-labeled secondary antibodies (Molecular Probes). Nuclei were also stained with DAPI (Molecular Probes). The fluorescent signals were visualized with an LSM 700 confocal laser scanning microscope (Carl Zeiss) equipped with 63/1.4 Oil DIC Plan-Apochromat objective lenses. Image processing was performed using the ZEN 2009 LSM software (Carl Zeiss).

3. Results and discussion

3.1. Ligand-induced auto-phosphorylation is severely impaired in all the mutants tested

Among the mutants prepared, 2 ($G^{589}E$ and $E^{633}K$) and 9 mutants ($M^{766}T$, $A^{770}P$, $I^{775}N$, $I^{794}T$, $D^{837}Y$, $F^{849}S$, $L^{868}P$, $M^{875}T$, and $P^{878}T$) possessed the amino acid substitutions in the ATP-binding site and major kinase domain, respectively (Fig. 1). However, irrespective of the mutation site, M-CSF stimulation failed to induce a detectable auto-phosphorylation at the tyrosine residue at a position of 809 (pY^{809}), in all the mutants (Fig. 1). This was also

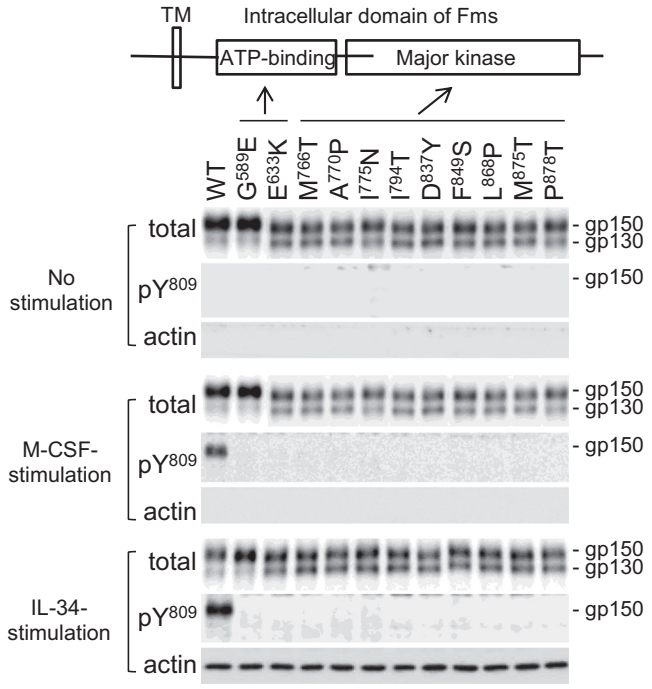

Fig. 1. The ligand-induced auto-phosphorylation of HDLS-related Fms mutants. The 11 Fms mutants used in this study are shown schematically. TM, transmembrane. The 293 cells stably expressing the wild-type (WT) or the indicated Fms mutant were stimulated with 100 ng/ml M-CSF or IL-34 at 37 °C for 5 min. Total cell lysates were subjected to Western blotting using antibodies against Fms (total) or Fms phosphorylated at Tyr809 (pY^{809}). The actin blot is a loading control. gp150 and gp130 indicate the high and low molecular weight Fms species, respectively. The data shown are representative of three independent experiments with similar results.

the case when the 293 cells expressing the mutant Fms proteins were stimulated with IL-34 (Fig. 1). Even in a more detailed time-course analysis (1, 10, 30, and 60 min) involving additional auto-phosphorylation sites such as pY^{546}, pY^{699}, pY^{708}, and pY^{723}, neither M-CSF (Fig. 2, blots) nor IL-34 (data not shown) induced detectable auto-phosphorylation signals in $G^{589}E$, $I^{775}N$, and $M^{875}T$ mutants. In contrast, both M-CSF and IL-34 markedly down-regulated the surface expression of the $G^{589}E$, $I^{775}N$, and $M^{875}T$ mutants (Fig. 2, bar graphs) and all of the remaining mutants (data not shown). As the binding of M-CSF leads to the down-regulation of cell surface Fms due to its internalization and lysosomal degradation [19], it appeared that the mutants tested had no major defect in the binding of M-CSF or IL-34, but a severe defect in the kinase activity.

3.2. Trafficking to the cell surface is also severely impaired in most mutants tested

Intriguingly, the $I^{775}N$ and $M^{875}T$ mutants showed decreased cell surface expression under the basal conditions (Fig. 2, bar graphs). Such decreased cell surface expression (Fig. 3, upper bar graph) and decreased M-CSF-binding (Fig. 3, lower bar graph) were also observed with $M^{766}T$, $A^{770}P$, $I^{794}T$, $D^{837}Y$, $F^{849}S$, $L^{868}P$, $P^{878}T$, and slightly with $E^{633}K$. The only the exception was $G^{589}E$, which was expressed at the surface at the level comparable to that of the wild-type (Fig. 3). Importantly, there was another difference between the wild-type and $G^{589}E$, and the remaining mutants, i.e., the absence or presence of a low molecular weight species (see Fig. 1). The species was a Fms-related product because it

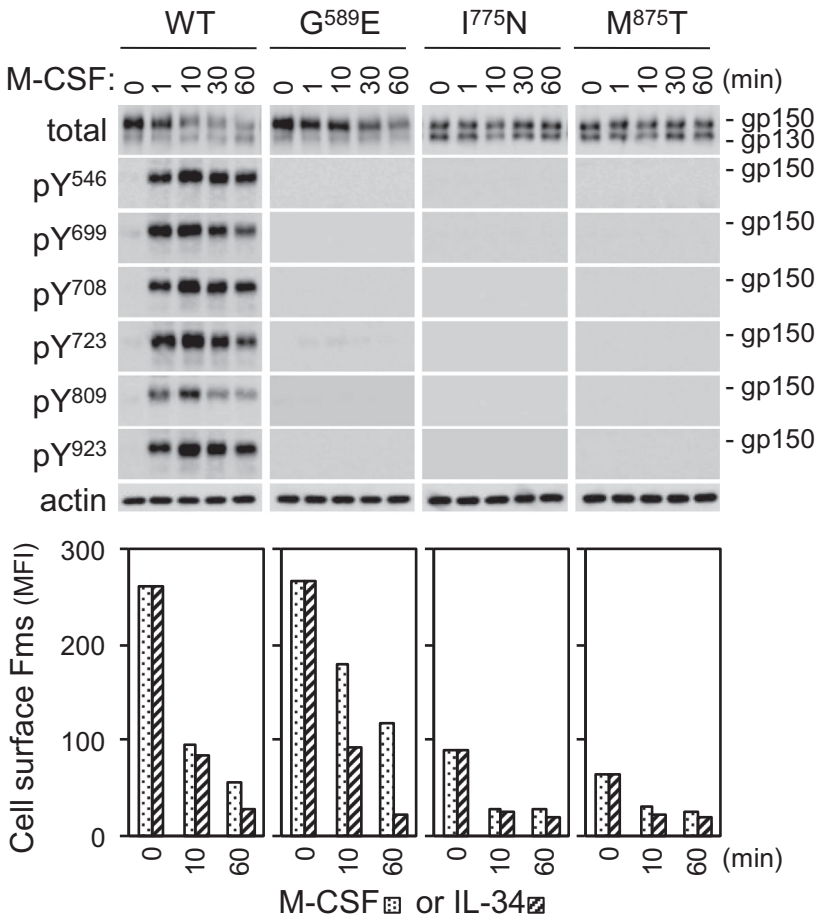

Fig. 2. The ligand-induced auto-phosphorylation and down-regulation of HDLS-related Fms mutants. In the upper blots, 293 cells stably expressing the wild-type (WT) or the indicated Fms mutant were stimulated with 100 ng/ml M-CSF for the indicated period at 37 °C. Total cell lysates were subjected to Western blotting. The following antibodies were used: anti-Fms (total) and anti-phosphorylated Fms (pY^{546}, pY^{699}, pY^{708}, pY^{723}, pY^{809}, and pY^{923}). The actin blot is a loading control. gp150 and gp130 indicate the high and low molecular weight Fms species, respectively. In the lower bar graphs, the cells were stimulated with 100 ng/ml M-CSF or IL-34 for the indicated period at 37 °C, and the cell surface expression of Fms was analyzed by flow cytometry. MFI, mean fluorescence intensity. The data shown are representative of three independent experiments with similar results.

was also detected by another anti-Fms antibodies (data not shown).

We previously found a similar change in Fms expression in a different experimental system [18,20,21]. When expressed in a myeloid cell line, the HIV-1 pathogenetic protein Nef impaired the response of the cells to M-CSF [20]. This was due to the decreased cell surface expression of Fms, but it was associated with a concomitant increase in the low molecular weight species [18,21]. Biochemical and immunological analyses revealed that the high molecular weight species (gp150Fms) was the fully *N*-glycosylated cell surface form whereas the low molecular weight species (gp130Fms) was the hypo-*N*-glycosylated immature form that accumulated to the Golgi-like perinuclear regions [18,21]. Due to similarities between the latter findings and those of the present study, we hypothesized that the HDLS-related Fms mutants other than $G^{589}E$ possessed a defect in the intracellular trafficking. A shown in Fig. 4, the wild-type and $G^{589}E$ showed clear signals at the cell surface together with additional vesicular signals which partially overlapped with the early endosome marker EEA-1. In contrast, $E^{633}K$, $F^{849}S$ and $P^{878}T$ (Fig. 4), and the remaining 7 mutants (data not shown) showed faint signals at the cell periphery but strong signals at perinuclear regions which partially overlapped with the Golgi marker GM130. The results indicated that most HDLS-related mutations caused a perturbed intracellular trafficking of Fms proteins.

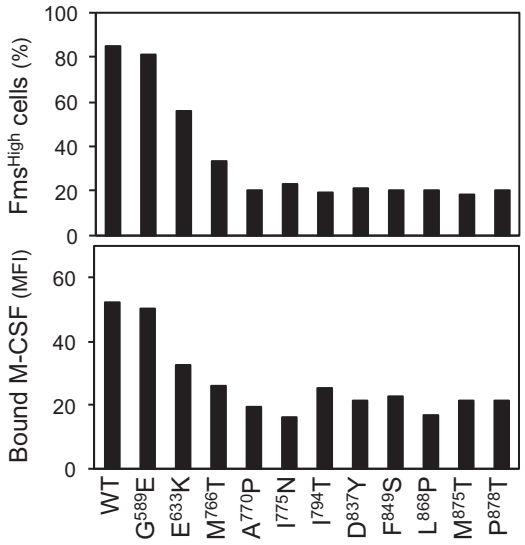

Fig. 3. The cell surface expression of HDLS-related Fms mutants. In the upper bar graph, 293 cells stably expressing the wild-type (WT) or the indicated Fms mutant were analyzed for their surface expression of Fms by flow cytometry. Data shown are the percentage of cells expressing Fms at a high level. In the lower bar graph, the binding of M-CSF-Flag proteins to these cells was analyzed by flow cytometry-based assays [7]. MFI, mean fluorescence intensity. The data shown are representative of three independent experiments with similar results.

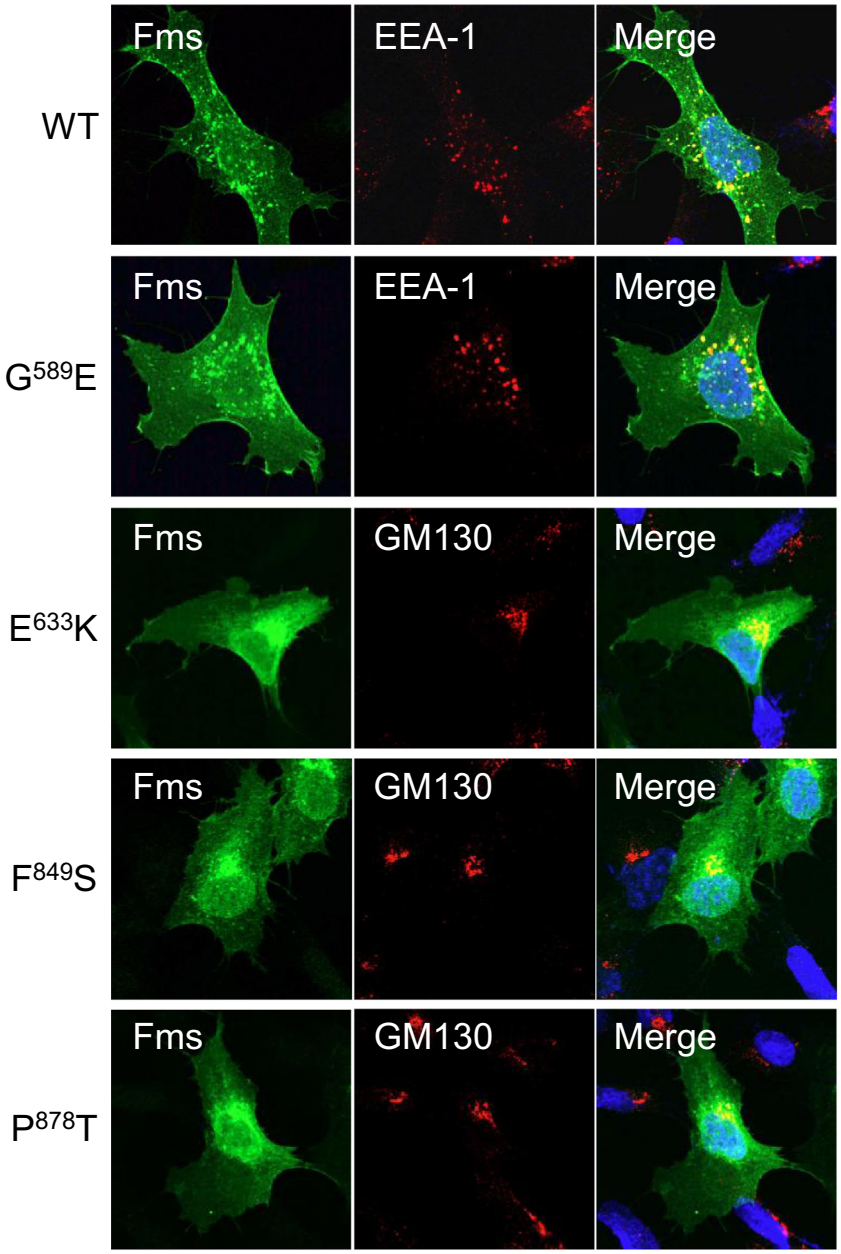

Fig. 4. The intracellular localization of Fms mutants. The 293 cells stably expressing the wild-type (WT) or the indicated Fms mutant were stained with anti-Fms antibodies (green). The cells were also stained with either anti-EEA-1 or GM130 (red), and DAPI (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The precise mechanism by which most HDLS-related mutations perturb Fms trafficking to the cell surface remains to be determined. At least, this was not simply due to severely impaired kinase activity because $G^{589}E$, of which kinase activity was also severely impaired (Figs. 1 and 2), had no apparent defect in the cell surface expression (Figs. 3 and 4). Thus, studies of HDLS-related mutations might help our understanding of how the maturation and intracellular trafficking of newly synthesized Fms and related receptor tyrosine kinases are physiologically regulated. In summary, we demonstrated that all of the Fms mutations tested severely impaired the kinase activity and most of the mutations also impaired the trafficking to the cell surface. These results imply that even if some of HDLS-related Fms mutants allow residual kinase activity, it is still difficult for M-CSF and IL-34 to transmit signals through the mutated receptors due to their impaired cell surface expression, further suggesting that HDLS is caused by a loss of Fms function.

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