Characterization of *mpl* Cytoplasmic Domain Sequences Required for Myeloproliferative Leukemia Virus Pathogenicity

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v-*mpl* is a truncated form of a receptor-like chain which belongs to the cytokine receptor superfamily. This sequence has been transduced in the myeloproliferative leukemia virus as an *env-mpl* fusion gene responsible for an acute myeloproliferative disorder in mice. We constructed a series of viral mutants in the *mpl* sequence. Analysis of their oncogenic potential in vivo indicated that a critical 69-amino-acid-long cytoplasmic domain of v-Mpl is required for myeloproliferative leukemia virus pathogenicity. We also developed an in vitro assay and showed that expression of the *env-mpl* gene confers growth factor independence to murine as well as to human hematopoietic growth factor-dependent cell lines. These findings strongly suggest that v-Mpl delivers a constitutive proliferative signal through a limited region of its cytoplasmic domain.

The myeloproliferative leukemia virus (MPLV) is a defective murine retrovirus which induces, in the presence of helper murine leukemia virus (MuLV), an acute myeloproliferative disorder with hepatosplenomegaly and polycythemia in all mouse strains (27). MPLV promotes in vitro growth and terminal differentiation of several hematopoietic precursor cells in the absence of growth factors (35, 36). MPLV originated from the transduction of a cellular sequence named *mpl* in frame with the partially deleted and rearranged envelope gene of Friend MuLV (F-MuLV) (31). Molecular cloning and expression analysis of the human and murine proto-oncogenes, namely, *h-mpl* and *m-mpl* (30, 33, 34), demonstrated that *c-mpl* encodes a hematopoietin receptor chain with a single transmembrane domain and characteristic motifs of the cytokine receptor superfamily. In v-Mpl, all but the last 43 amino acids (aa) COOH terminal of the 483 extracellular aa of m-Mpl have been deleted. The entire transmembrane and cytoplasmic domains are identical to those of m-Mpl except for the 2 COOH-terminal aa which are absent in v-Mpl (31, 33). In this study, we analyzed more precisely the cytoplasmic regions of the *env-mpl* oncogene involved in MPLV pathogenicity.

Comparison of the cytoplasmic sequences of m-Mpl and v-Mpl has shown that v-Mpl differs from m-Mpl only by the absence of the last 2 COOH-terminal aa of m-Mpl (a glutamine and a proline). These 2 aa were reintroduced into the MPLV genome by exchanging a 188-nucleotide (nt) *NcoI-BclI* fragment between m-*mpl* and v-*mpl* after a *BclI* restriction site had been created at the v-*mpl* *StuI* site (Fig. 1). Since MPLV does not transform NIH 3T3 cells, the biological activity of the modified MPLV, called 2m-MPLV, was tested in vivo. 2m-MPLV was cotransfected with helper F-MuLV clone 57 (26) into NIH 3T3 cells at a molar ratio of 10:1 (2m-MPLV/F-MuLV). As a positive control, an infectious wild-type MPLV molecular clone was cotransfected with F-MuLV under the same conditions, and F-MuLV alone was transfected as a negative control. Cells were passed four times at confluency. Two weeks after transfection, viral production was tested by reverse transcriptase assay. The presence of the two viral

genomes, 2m-MPLV and F-MuLV, was verified by the Hirt method, using a *gag* probe which hybridized to both F-MuLV and MPLV (16). 2m-MPLV and F-MuLV genomes were present in equal proportions, as observed with the wild-type MPLV isolate (data not shown). Viral supernatants (0.5 ml) were injected intravenously into 2-month-old ICFW female mice. Mice displaying gross organ enlargement were bled for hematocrit determination and were sacrificed. Spleen weight values as well as spleen imprints and blood smears were examined for each mouse. As indicated in Fig. 2, all of the eight mice inoculated with 2m-MPLV developed a typical MPLV pathology, including splenomegaly and polyglobuly characteristic of this disease, while mice inoculated with helper F-MuLV did not. This result indicates that the absence of the 2 COOH-terminal aa of m-Mpl in v-Mpl is not required for the pathogenicity of MPLV.

We tested whether replacement of the v-Mpl sequence by its human homolog would alter MPLV pathogenicity. To address this question, we replaced almost all the v-*mpl* sequence by the corresponding human region by exchanging a 443-nt *PvuII-NcoI* fragment of v-*mpl* for the corresponding fragment of h-*mpl* (Fig. 1). The resultant construct, called h-MPLV, exhibits 18 aa differences from wild-type MPLV, 11 of which are located in the cytoplasmic domain. This construct was tested in the same conditions as 2m-MPLV. h-MPLV induced a myeloproliferative syndrome in mice with the same characteristics as that induced by wild-type MPLV (Fig. 2). These data show that h-Mpl sequences are functional in a murine background and that amino acid substitutions introduced in h-MPLV do not alter MPLV pathogenicity.

We also generated several deletion mutants of v-*mpl* and reintroduced them into the MPLV genome (Fig. 1). All v-*mpl* mutants were made in the pBluescript KS+ vector (Stratagene), in which a 1,068-nt *SacII-ClaI* fragment from the *env* region of MPLV had been cloned (*env-mpl*-pBluescript vector). Mutants SC-MPLV, SS-MPLV, and Δ4-MPLV were generated by introducing termination codons (including a *XbaI* site) at the *CellI*, *SphI*, and *NcoI* restriction sites, respectively. Mutants ΔTM-MPLV, Δ1-MPLV, Δ2-MPLV, and Δ3-MPLV were generated by site-directed mutagenesis as previously described (1). Entirely sequenced mutated fragments were reintroduced into the *env-mpl*-pBluescript vector, and the mutated *SacII-ClaI* fragments were reinserted into the unique

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FIG. 1. *env-mpl* constructs assayed for pathogenicity. (A) Schematic structures of wild-type (wt) and mutated Env-Mpl fusion proteins. Restriction sites of the *env-mpl* region used for mutagenesis and cloning are shown. The rectangle depicts the coding region. (*SacII*) indicates the site modified for cloning in the pBabeneo vector. EC, extracellular domain; IC, intracellular domain; TM, transmembrane domain (shown as a black box). The long hatched box represents the Env region, and the small hatched box indicates the WS motif very well conserved in the cytokine receptor family. The positions of boxes 1 and 2 are also specified. The 2m-MPLV mutant contains the COOH-terminal glutamine and proline residues from the murine c-Mpl. In h-MPLV, the stippled area indicates substitution of the human counterpart in v-Mpl. The deletions introduced in each mutant are represented by dotted lines. Positions of the first deleted amino acids of mutants are indicated. (B) Pathogenicity of MPLV and mutants.

SacII and *ClaI* sites of the MPLV molecular clone. All mutant viruses were produced and tested as indicated above. Only two of these mutants, $\Delta 3$ and $\Delta 4$ -MPLV, were pathogenic (Fig. 2), indicating that the 50 COOH-terminal aa of v-Mpl are dispensable for the oncogenicity of v-Mpl in vivo. Three months after inoculation, mice presented only little increase in spleen weight, as did mice inoculated with F-MuLV alone. Differences in viral titers did not account for the lack of pathogenicity of the latter mutants since by Hirt analysis, the F-MuLV/MPLV ratio was of the same order of magnitude for all MPLV mutants (data not shown). Furthermore, we developed an infectivity assay and detected MPLV viral DNA by PCR on DNA from NIH 3T3 cells infected by endpoint viral dilutions. To specifically amplify the Env-Mpl sequence, we used the 5' oligonucleotide in the gp70 region and the 3' oligonucleotide in the Mpl cytoplasmic region, as previously described (1). All stocks contained between 5×10^4 and 5×10^5 infectious MPLV particles per ml (data not shown). Finally, mice were inoculated with log dilutions of wild-type MPLV. Although a slight increase in the appearance of a splenomegaly was observed, all mice inoculated with dilutions containing as few as 5×10^2 infectious MPLV particles per ml developed a myeloproliferative syndrome.

To confirm the data obtained in vivo, we took advantage of the ability of MPLV to abrogate the growth factor requirement of hematopoietic precursor cells (35, 36) to develop an in vitro assay for *env-mpl* activity in hematopoietic cell lines. For this purpose, we cloned a 1,068-nt *SacII* fragment encompassing the *env-mpl* sequence (Fig. 1) into the pBabeneo retroviral vector (*env-mpl*-pBabeneo vector) (23). In this vector, the *env-mpl* gene is expressed from the 5' long terminal repeat and

expression of the neomycin phosphotransferase gene is driven by an internal simian virus 40 promoter. We first wondered whether v-Mpl could transduce a proliferative signal in human cells. To address this question, we used the UT-7 human leukemic cell line (20), which is dependent for growth on interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor, or erythropoietin. UT-7 cells were electroporated with 20 μ g of the *env-mpl*-pBabeneo vector or the pBabeneo vector alone. After 1 week of G418 selection (800 μ g/ml), colony formation was assayed in the absence of growth factor in medium containing 1% methylcellulose. Colonies appeared only after electroporation with the *env-mpl*-pBabeneo vector. Individual colonies were picked and expanded in liquid culture. Four clones proliferating in the absence of recombinant human granulocyte-macrophage colony-stimulating factor were analyzed by Northern (RNA) blotting. They all exhibited a high expression of the *env-mpl* gene (Fig. 3). Therefore, the murine cytoplasmic region of Mpl, present in the *env-mpl* fusion gene, can trigger the growth factor-independent proliferation of a human cell line.

We next assayed the murine myeloid FDC-P1 cell line, which is strictly dependent on the presence of IL-3 for its proliferation (5). As for the UT-7 cell line, FDC-P1 cells became growth factor independent when transfected with the *env-mpl*-pBabeneo vector but not after transfection with the pBabeneo vector. To support our results obtained in vivo, constructs which were nonpathogenic (Δ TM, $\Delta 1$, and $\Delta 2$ *env-mpl*) and one pathogenic mutant ($\Delta 3$ *env-mpl*) were subcloned in the pBabeneo vector. After 2 weeks of selection with G418 (600 μ g/ml), Northern blot analysis confirmed the expression of the *env-mpl* gene of the various constructs in

FIG. 2. Pathogenicity assay of MPLV constructs. For each virus, five to eight mice were inoculated with undiluted viral supernatant obtained from transfected NIH 3T3 cells. Mice were sacrificed 3 to 6 weeks after inoculation. (A) Individual spleen weights; (B) hematocrit. S.E.M., standard error of the mean.

transfected cells (Fig. 3). Wild-type or Δ3 *env-mpl* transfectants exhibited total independence of IL-3, while in the ΔTM, Δ1, and Δ2 *env-mpl* or pBabeneo vector transfectants, more than 95% of the cells could be stained by trypan blue 24 h after IL-3 deprivation. This in vitro assay therefore confirmed our in vivo findings, indicating that the 69 juxtamembrane aa of Mpl are absolutely required for its biological activity.

Although homology between m-Mpl and h-Mpl is very high (86% at the amino acid level), differences in sequences do exist, including 11 aa in their cytoplasmic domains. In the 69-aa juxtamembrane domain which is absolutely required for Mpl biological activity, 5 aa, comprising three conservative exchanges and two nonconservative substitutions, differ: a proline for a serine and an arginine for a serine (at positions 37 and 64, respectively, when numbering starts at the first cytoplasmic amino acid). These differences do not confer any species specificity, nor do they alter MPLV pathogenicity. Furthermore, this cytoplasmic subdomain contains two motifs conserved in the cytokine receptor superfamily, namely, boxes 1 and 2, which are 100% homologous between h-Mpl and m-Mpl. The box 1 motif is present in all cytokine receptor-transducing chains (Fig. 4). In all receptors examined thus far,

FIG. 3. v-Mpl expression in hematopoietic cells electroporated with the *env-mpl*-pBabeneo vector. Fifteen micrograms of total RNA prepared by the guanidium isothiocyanate-cesium chloride method was denatured in glyoxal buffer, electrophoresed, blotted onto a nitrocellulose membrane, and hybridized with radiolabeled probes (random-primed DNA labeling kit; Boehringer). Hybridization was performed with an Mpl-specific probe and an actin probe, as indicated. (A) Expression in UT-7 cells. Sources of RNAs were control UT-7 cells (lane 1) and four different clones of UT-7 cells electroporated with the *env-mpl*-pBabeneo vector (lanes 2 to 5). (B) Expression in FDC-P1 cells. Sources of RNAs were control FDC-P1 cells (lane 1), FDC-P1 cells electroporated with the pBabeneo vector alone (lane 2), FDC-P1 cells electroporated with wild-type *env-mpl*-pBabeneo vector (lane 3), or Δ1, Δ3, and ΔTM mutants (lanes 4 to 6, respectively).

FIG. 4. Comparison of conserved motifs in cytokine receptors. (A) Box 1; (B) box 2. Numbers in parentheses indicate amino acid residue positions distal to the transmembrane domain when numbering starts at the first cytoplasmic residue. Letters in solid boxes indicate identity, and letters in dotted boxes denote conservative substitutions. Representative sequences shown: v-Mpl (31), h-Mpl (34), human and murine IL-7R (hIL-7R and mIL-7R) (10), human and murine IL-2R β chain (h-IL-2R β and mIL-2R β) (13, 21), human and murine erythropoietin receptor (hEPOR and mEPOR) (4, 19), murine IL-3R-specific β chain or AIC2A (18), murine and human β chains common to the IL-3R, granulocyte-macrophage colony-stimulating factor receptor, and IL-5R or AIC2B and KH97, respectively (11, 14), human and murine IL-4R (hIL-4R and m-IL-4R) (17, 24), human and murine signal-transducing β chain of the IL-6R or gp130 (hgp130 and mgp130) (15, 28), human and murine granulocyte colony-stimulating factor receptor (hG-CSFR and mG-CSFR) (7, 8), human leukemia inhibitory factor receptor (hLIFR) (9), human prolactin receptor (hPRLR) (2), and human growth hormone receptor (hGHR) (22).

it appears to be absolutely required for signal transduction (3, 6, 25). The box 2 motif is present in most cytokine receptors (Fig. 4), but its presence is not always required for the proliferative signal, depending on receptors and/or cells used for assays (25, 29, 37). In the $\Delta 1$ -MPLV mutant, which lacks box 1 but contains box 2, the pathogenicity was completely abolished. The same results were obtained with the $\Delta 2$ -MPLV mutant, in which box 1 was conserved but box 2 was deleted. Apart from boxes 1 and 2, clusters of acidic residues are frequently present in the cytoplasmic domains of cytokine receptors. In the IL-2 receptor (IL-2R) β chain and IL-7R, acidic regions are involved in the interaction with kinases of the Src family (12, 32). An acidic cluster overlapping box 2 also exists in the Mpl cytoplasmic sequence (6 acidic out of 19 aa). Whether the Mpl acidic cluster interacts with cytoplasmic molecules such as Src kinases is under investigation. Interestingly, distinct cytoplasmic regions of a receptor may be involved in different cellular signals. For example, the human granulocyte colony-stimulating factor receptor requires only the 57 first cytoplasmic aa encompassing boxes 1 and 2 to transduce a proliferative signal in the pro-B BAF/B03 cell line, while 40 more COOH-terminal residues are required for the induction of specific gene expression in response to granulocyte colony-stimulating factor in human hepatoma cells (6, 37). By analogy, as v-Mpl does not block terminal differentiation of hematopoietic precursor cells, it will be of particular interest to study whether differentiation and proliferation are specified by the same subdomains of Mpl.

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