Constitutive Activation of a Variant of the *env-mpl* Oncogene Product by Disulfide-Linked Homodimerization

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The myeloproliferative leukemia retrovirus (MPLV) has the *v-mpl* cellular sequences transduced in frame with the deleted and rearranged Friend murine leukemia virus *env* gene. The resulting *env-mpl* fusion oncogene is responsible for an acute myeloproliferative disorder induced in mice by MPLV. *v-mpl* is a truncated form of the *c-mpl* gene which encodes the receptor for thrombopoietin. We investigated the contribution of the Env-Mpl extracellular domain in the constitutive activation of this truncated cytokine receptor and found that the rearrangement of the *env* sequences in the *env-mpl* fusion gene was not required for oncogenicity. A pathogenic variant, DEL3MPLV, was generated, which differs from MPLV by the deletions of 22 amino acids of the Env signal peptide, all of the mature Env sequences, and 18 N-terminal amino acids of the v-Mpl extracellular domain. The resulting *del3-mpl* oncogene product conserves in its extracellular region the first 12 amino acids of the Env signal sequence including a cysteine residue, and 25 amino acids of the v-Mpl. We show here that a mutation converting this cysteine to a glycine completely abolishes *del3-mpl* oncogenicity and that the *del3-mpl* oncogene product is constitutively activated by disulfide-linked homodimerization.

Myeloproliferative leukemia retrovirus (MPLV) is a defective murine retrovirus generated during the *in vivo* passage of a molecularly cloned Friend murine leukemia virus (F-MuLV) helper virus (F-MuLV 57). In the presence of helper virus, MPLV induces an acute myeloproliferative disease characterized by hepatosplenomegaly with myeloid hyperplasia and polycythemia (23). Hematopoietic progenitor cells from diseased mice are growth factor independent for both proliferation and terminal differentiation. Moreover, direct *in vitro* infection of normal bone marrow cells by MPLV leads to the generation of cell lines of various hematopoietic lineages, and infection of growth factor-dependent hematopoietic cell lines abrogates their growth factor requirement (38, 39). The original MPLV isolate encodes retroviral *gag* and *pol* sequences derived from F-MuLV and an *env-mpl* fusion gene which is responsible for MPLV biological properties (1). *v-mpl* is derived from a unique cellular sequence encoding a truncated form of a cytokine receptor which has been transduced in frame into the partially deleted F-MuLV *env* gene (29). Human and mouse *c-mpl* proto-oncogenes have been cloned (27, 32, 33) and their products have been shown to be members of the hematopoietin receptor superfamily characterized by common structural features: two pairs of cysteines and a WSXWS box in their extracellular domain (1), a single transmembrane region, and a cytoplasmic domain devoid of a tyrosine protein kinase consensus sequence (20, 26). Recently, c-Mpl was demonstrated to be the receptor for a new cytokine, thrombopoietin, which promotes megakaryocyte progenitor proliferation and stimulates platelet production *in vivo* (5, 12, 15, 37). The fragment of *c-mpl* transduced in MPLV consists of sequences encoding the cytoplasmic and transmembrane regions, but most of the c-Mpl extracellular domain has been deleted. Since the Env-Mpl protein most probably functions as a constitu-

tively activated receptor, we studied the functional regions of this oncogene involved in signal transduction. We previously showed that the 69 juxtamembrane cytoplasmic amino acids and the transmembrane region of v-Mpl are required for the transmission of a proliferative signal (2), while the WSXWS box of the Env-Mpl extracellular domain is not involved in MPLV pathogenicity (1). In the present study, we investigated the role of the Env-Mpl extracellular domain, which is composed of 100 N-terminal rearranged residues of gp70 fused to 43 amino acids of the v-Mpl sequence (29). To examine the potential role of the internal *env* deletion in *v-mpl* activation, we reinserted F-MuLV *env* sequences coding for the 179 amino acids that were deleted in the *env-mpl* product in a fusion gene designated *sup-mpl*. We show here that the initial deletion of the *env* gene is not required for MPLV pathogenicity. An oncogenic MPLV variant, DEL3MPLV, was generated *in vivo* in which the oncogene did not contain any Env sequences except for the 12 N-terminal residues of the 34 amino acids constituting the Env signal peptide. The protein encoded by this oncogene, Del3-Mpl, was shown to form covalent homodimers. We demonstrated that, unlike in the original MPLV isolate, activation of the *v-mpl* sequences in DEL3MPLV involves disulfide-linked homodimerization.

MATERIALS AND METHODS

Construction and production of the virus SUPMPLV. The *sup-mpl* fusion gene was constructed by exchanging a 293-nucleotide (nt) *Bam*HI fragment of the *env-mpl* gene for an 830-nt *Bam*HI fragment of the *env* F-MuLV 57 corresponding region. The SUPMPLV proviral DNA was constructed by subcloning a *Sac*II-*Cla*I fragment of the *sup-mpl* gene into the unique *Sac*II and *Cla*I sites of the MPLV molecular clone. SUPMPLV DNA was cotransfected with helper F-MuLV into NIH 3T3 cells at a molar ratio of 10:1. Cells were passaged four times at confluency, and viral production was tested in cell supernatant by reverse transcription assay. The presence of the two viral genomes was verified by the Hirt method.

Mouse infection and survey. Viral supernatants (0.5 ml) were injected intravenously into 6-week-old DBA/2 female mice. Pathogenesis was monitored by regular spleen palpation. Mice displaying gross organ enlargement were bled for hematocrit determination and were sacrificed. Spleen weights as well as spleen imprints and blood smears were examined for each mouse.

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FIG. 1. Env-Mpl fusion proteins encoded by SUPMPLV, MPLV, and DEL3MPLV. (A) Schematic representation of SUPMPLV provirus. The positions of *EcoRI* and *ClaI* restriction sites used for Southern blot analysis are indicated. Thick bars represent the *sup-mpl* oncogene as an *XbaI*-*ClaI* fragment. Cleavage sites for the endonucleases used for cloning and mutagenesis are shown. The positions of the oligonucleotides used for *del3-mpl* analysis and cloning are indicated at the bottom. (B) Structure of Sup-Mpl, Env-Mpl, and Del3-Mpl proteins. Restriction sites of *sup-mpl* in panel A line up with the domains of Sup-Mpl in panel B. Crosshatched boxes represent Env sequences (in Sup-Mpl and Env-Mpl, amino acids 1 to 34 form a signal peptide). White boxes represent *mpl*-specific sequences, and black boxes indicate the transmembrane (TM) domain. Dotted lines delimit deleted regions: Env amino acids 65 to 243 are deleted in MPLV; 22 amino acids of the signal peptide, mature Env sequences, and 18 N-terminal amino acids of the v-Mpl are deleted in DEL3MPLV. Solid circles indicate the positions of cysteine, and the asterisk represents the point mutation that converts threonine 15 into a proline in DEL3MPLV. aa, amino acids.

PCR amplification and analysis of provirus integrated in the spleens of diseased mice. PCR was performed on spleen genomic DNA by using two sets of primers flanking the region encoding the Sup-Mpl extracellular domain (Fig. 1A), two 5' primers (primer a [5'-ACGCTGCCCACGTAAAGGCT] and primer c [5'-TCTCAAAGGGGCCAGATC]) and two 3' primers (primer b [5'-AGTGCCCTTCTGGGCTACT] and primer d [5'-TGGTGACTGCTCTGCTCCTG GT]). PCR was carried out with AmpliTaq (Perkin-Elmer Cetus) by using a thermocycler (Perkin-Elmer Cetus) under reaction conditions recommended by Cetus. DNAs were analyzed by gel electrophoresis and blotted onto nylon membrane (Hybond N⁺; Amersham) for hybridization with the *mpl* *SacI*-*StuI* probe (2).

Molecular cloning of the *del3-mpl* oncogene and reconstruction of DEL3MPLV provirus. The region of *del3-mpl* encoding the extracellular domain was cloned by PCR amplification performed on spleen genomic DNA by using primers a and b.

Transmembrane and cytoplasmic regions were cloned by PCR amplification with 5' primer e (5'-TGGTGACTGCTCTGCTCCTGGT) and 3' primer f (5'-CCTGAAGGCAGTCCCCATG). All the PCR fragments were cloned in pBlue-script II KS, and nucleotide sequences were determined on both strands by the dideoxynucleotide chain-terminating method with the T7 sequencing kit (Pharmacia).

To construct the DEL3MPLV molecular clone, a *Bam*HI-*Cel*II cassette of the *del3-mpl* (encoding the extracellular domain) was exchanged for the *Bam*HI-*Cel*II cassette of the *env-mpl* gene. The *del3-mpl* gene was inserted as an *XbaI*-*ClaI* fragment into MPLV DNA digested with *ClaI* and partially digested with *XbaI*.

Mutagenesis techniques. The mutants *T15del3-mpl* and *G3del3-mpl* were generated by PCR with synthetic oligonucleotide primers encoding the chosen amino acid substitutions. To mutate proline 15, an A-to-T substitution was generated at nucleotide 44 of the *del3-mpl* coding sequence. To mutate cysteine 3, we carried out the transition T to G at nucleotide 7 of the *del3-mpl* sequence. An epitope tag derived from the G protein of vesicular stomatitis virus (REG PPGPYTDIEMNRLGK) recognized by the P5D4 monoclonal antibody (MAb) (28) was introduced at the C-terminal ends of Env-Mpl, Del3-Mpl, and mutants

in place of the 50 cytoplasmic C-terminal residues of v-Mpl which had been shown to be dispensable for proliferation (2). These constructs were obtained by PCR techniques, and their sequences were confirmed by the dideoxynucleotide chain-terminating method.

Cells and electroporation. The *env-mpl*, *del3-mpl*, *T15del3-mpl*, and *G3del3-mpl* genes were subcloned into a modified pRSV expression vector (Invitrogen Corporation) as *SalI* fragments. Interleukin-3 (IL-3)-dependent murine FDC-P1 cells were grown in minimal essential medium alpha supplemented with 10% fetal calf serum and 6% WEHI-3B-conditioned medium as a source of IL-3. Approximately 10⁷ cells were electroporated with the constructs (50 µg) in a Bio-Rad electroporator set at 960 µF and 250 V. Cells were plated in a microtiter plate and selected for 2 weeks in medium containing 0.5 mg of G418 per ml. Growth factor independence was assayed by total deprivation of WEHI medium for each clone.

Protein extraction. Cells were solubilized with Nonidet P-40 lysis buffer (1% Nonidet P-40, 20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride, 1 trypsin inhibitor unit of aprotinin per ml and 10 µg of leupeptin per ml. Clear lysates obtained by centrifugation were incubated with the P5D4 MAb (1:200 dilution). The immunoprecipitates were absorbed with fixed *Staphylococcus aureus*, washed, eluted, and subjected to electrophoresis on a sodium dodecyl sulfate (SDS)-12% polyacrylamide gel followed by Western blotting (immunoblotting).

Western blot analysis. SDS-polyacrylamide gel electrophoresis (PAGE) gels were transferred to nitrocellulose. Nitrocellulose membranes were blocked with 5% fat-free milk and incubated overnight with P5D4 MAb (1:1,000 dilution). Proteins were detected with peroxidase-linked sheep anti-mouse antibody and by chemiluminescence (ECL kit; Amersham).

RESULTS

Role of *env* sequence deletion in MPLV pathogenicity. The *env-mpl* fusion gene encodes a protein of 284 amino acids

FIG. 2. Pathogenicity assays of F-MuLV 57, MPLV, SUPMPLV, and DEL3MPLV. For each virus three to eight mice were inoculated with undiluted viral supernatant obtained from transfected NIH 3T3 cells. Mice were sacrificed 5 to 12 weeks after inoculation. (A) Individual spleen weights; (B) individual hematocrits.

containing 100 N-terminal residues derived from rearranged F-MuLV 57 Env sequences: Env residues 1 to 64 (including the initiation codon and the 34 amino acids of the signal peptide) are fused to Env residues 244 to 280, in frame with the 43 extracellular, 22 transmembrane, and 119 cytoplasmic amino acids of the v-Mpl sequence (Fig. 1B).

To determine whether the internal *env* deletion had any role in MPLV pathogenicity, the F-MuLV *env* region encoding amino acids 65 to 243 was reinserted into the *env* portion of the *env-mpl* fusion gene as described in Materials and Methods. In this construct, the N-terminal 280 amino acids of F-MuLV Env are fused to v-Mpl sequences (Fig. 1B). This provirus construct was designated SUPMPLV. The biological activity of this virus was tested in vivo. SUPMPLV DNA was cotransfected with helper F-MuLV 57 into NIH 3T3 cells. As a positive control, a wild-type MPLV molecular clone was cotransfected with F-MuLV under the same conditions, and F-MuLV alone was transfected as a negative control. Viral production was tested by reverse transcription assay, and the presence of viral genomes was determined by the Hirt method with a *gag* probe which hybridized to F-MuLV, MPLV, and SUPMPLV genomes. Mice were then inoculated with viral supernatant, and pathogenesis was monitored as described in Materials and Methods. Mice inoculated with MPLV or SUPMPLV developed a myeloproliferative disease characterized by hepatosplenomegaly, polyglobuly, thrombocytosis, and granulocytosis

(Fig. 2). However, the latency of the disease induced by SUPMPLV (8 to 12 weeks) was significantly longer than that observed with MPLV (3 to 5 weeks). To examine the structure of SUPMPLV proviruses integrated in enlarged spleens of diseased mice, Southern blot analyses were performed on genomic DNA digested with the *EcoRI* and *ClaI* endonucleases whose cleavage sites flank the *sup-mpl* gene (Fig. 3A). Hybridization with an *mpl*-specific probe would detect an internal 4,589-nt fragment in SUPMPLV proviral DNA. This analysis revealed no gross rearrangement of the *sup-mpl* fusion gene in most of the inoculated mice (23 of 24). Moreover, PCR amplification was performed with 23 spleen DNAs with two sets of primers, a-b and c-d (Fig. 1A), flanking the region encoding the Sup-Mpl extracellular domain. This analysis indicated that for all mice examined, fragments of the expected sizes (1,080 and 911 nt for the a-b and c-d primer sets, respectively) were specifically amplified (data not shown). All these results indicate that the SUPMPLV virus is pathogenic and that in the *env-mpl* fusion oncogene, the deletion within the *env* gene is dispensable for MPLV pathogenicity.

Analysis of deletion mutant DEL3MPLV. Southern blot analysis performed on spleen genomic DNA showed that in 1 of 24 diseased mice infected with SUPMPLV, a large deletion in the *env-mpl* gene had occurred (Fig. 3B). In fact, instead of the expected 4.6-kb *EcoRI-ClaI* fragment, a 3.7-kb fragment was detected as a major hybridizing band. This deletion was

FIG. 3. Southern blot analysis of proviruses integrated in spleens of diseased mice. Genomic DNA was isolated from the enlarged spleens of diseased mice, digested with *Eco*RI and *Cla*I restriction endonucleases, resolved on a 1% agarose gel, and blotted and hybridized with an *mpl*-specific probe. Positions of SUPMPLV-, MPLV-, DEL3MPLV-, and *c-mpl*-specific hybridizing fragments are indicated by arrows. (A) Mice 1 to 8 were inoculated with SUPMPLV (lanes 1 to 8, respectively). DEL3MPLV was generated in mouse 7. (B) Mouse 9 was inoculated with MPLV (lane 9); lanes 6 and 7 are the same as in panel A.

even larger than that in MPLV, which yielded, after digestion, a 4,052-nt *Eco*RI-*Cla*I fragment. PCR amplification of DNA derived from this mouse, with a sense oligonucleotide starting at position 1 of the *env* gene and an antisense oligonucleotide located in *v-mpl* encoding the transmembrane region, generated a 220-nt fragment, indicating that the deletion occurred in the extracellular-domain-encoding region of the *env-mpl* gene (the same primers allow the amplification of a 1,078-nt PCR product fragment in *sup-mpl* proviral DNA). We cloned this PCR fragment and sequenced four independent clones. We found that this new provirus, DEL3MPLV, had deleted the 22 C-terminal amino acids of the signal peptide, including its hydrophobic core and all of the mature Env sequences. Eighteen N-terminal amino acids of the v-Mpl extracellular domain were also deleted in DEL3MPLV, and a point mutation had changed a threonine into a proline at position 15 of the Del3-Mpl amino acid sequence (Fig. 1B). Thus, the Del3-Mpl extracellular domain is composed of the first 12 N-terminal amino acids of the Env signal peptide fused to the 25 juxtamembrane residues of the v-Mpl extracellular domain. We have also cloned the transmembrane and cytoplasmic regions of this new provirus and found that it did not contain additional mutations. A molecular clone of DEL3MPLV was constructed, pseudotyped with F-MuLV, and found to be as oncogenic as wild-type MPLV (disease latency of 3 to 5 weeks) in vivo.

Roles of the proline 15 mutation and cysteine 3 in the DEL3MPLV extracellular domain. The 12 N-terminal amino acids of the signal sequence fused to v-Mpl in DEL3MPLV contain a cysteine at position 3 of the Del3-Mpl amino acid sequence. Assuming that these 12 amino acids do not form a functional signal sequence, we wondered whether cysteine 3 and the threonine-to-proline mutation at position 15 had roles in the constitutive activation of *del3-mpl*. For this purpose, these amino acids were independently mutated as described in Materials and Methods. Threonine 15 was restored in T15Del3-Mpl, and cysteine 3 was mutated to glycine in G3Del3-Mpl. To better analyze the expression of the Env-Mpl, Del3-Mpl, and mutant proteins, epitope-tagged derivatives were constructed as described in Materials and Methods. The inability of MPLV to transform NIH 3T3 cells precludes an

easy titration of this defective virus. Since we had previously shown that the pathogenicity of MPLV mutants correlated with their ability to abrogate the growth factor requirement of hematopoietic cells in vitro (2), the biological properties of the mutated genes were tested in vitro. The constructs *env-mpl-tag* and *del3-mpl-tag* and the mutated genes *T15del3-mpl-tag* and *G3del3-mpl-tag* were subcloned into the expression vector pRSVNeo and electroporated into the murine myeloid IL-3-dependent FDC-P1 cell line (7). The vector alone was electroporated under similar conditions. Electroporated cells were selected with medium containing G418, and single G418-resistant clones were tested for IL-3-independent growth. We found that 8 of 10 FDC-P1 clones expressing *env-mpl-tag*, 13 of 16 clones expressing *del3-mpl-tag*, and 6 of 6 clones expressing *T15del3-mpl-tag* were growth factor independent after neomycin selection (Table 1). In contrast, none of the FDC-P1 clones containing the pRSVNeo vector and none of the 40 clones transfected with the *G3del3-mpl-tag* construct were growth factor independent. Two clones of each series were subsequently expanded for protein analysis; clones expressing *G3del3-mpl-tag* or transfected with the pRSVNeo vector were cultured in the presence of IL-3 and G418, while all other clones were

TABLE 1. FDC-P1 electroporation with different vectors^a

Vector (expt no. ^b)	No. of clones	
	Neo ^r	Growth factor independent
pRSVNeo alone (1)	6	0
pRSVNeo alone (2)	4	0
<i>env-mpl-tag</i> (1)	10	8
<i>del3-mpl-tag</i> (1)	6	6
<i>del3-mpl-tag</i> (2)	10	7
<i>T15del3-mpl-tag</i> (1)	6	6
<i>G3del3-mpl-tag</i> (1)	20	0
<i>G3del3-mpl-tag</i> (2)	20	0

^a Cells were electroporated with vector and were selected in a microtiter plate in medium containing G418. Each G418-resistant clone was tested for IL-3-independent growth.

^b Two independent experiments were performed.

FIG. 4. V-Mpl protein expression in transfected FDC-P1 cells. Parental FDC-P1 cells (control) or FDC-P1 cells transfected with *G3del3-mpl-tag*, *del3-mpl-tag*, and *T15del3-mpl-tag* were expanded for protein analysis after G418 selection. Nonidet P-40 lysates from 20×10^6 cells were subjected to immunoprecipitation with the P5D4 MAb, and immunoprecipitates were analyzed by SDS-12% PAGE and subsequent immunoblotting with the P5D4 MAb. IgL, immunoglobulin light chain.

cultured without IL-3. As shown in Fig. 4, Mpl-Tag proteins were detectable in transfected cells, although at low levels in most of the clones. These results demonstrate that the presence of cysteine 3 is necessary while the proline 15 mutation is dispensable for *del3-mpl* constitutive activation.

Disulfide-linked homodimerization of Del3-Mpl. The requirement of cysteine 3 prompted us to determine whether this residue was involved in a disulfide-linked bridge. Cell lysates prepared from a growth factor-independent FDC-P1 clone with the highest level of expression of Del3-Mpl-Tag protein were treated with the anti-Tag MAb (P5D4), and immunoprecipitates were electrophoresed under reducing or nonreducing conditions and then subjected to Western blotting analysis. This experiment showed that under reducing conditions the Del3-Mpl-Tag protein was expressed as an 18-kDa band, a relative molecular mass that agrees well with the predicted size. By contrast, under nonreducing conditions, the Del3-Mpl-Tag protein ran as two bands, an 18-kDa band indistinguishable in size from the band found under reducing conditions

and a 36-kDa band which presumably corresponds to a dimer (Fig. 5A). This result together with the requirement for a cysteine residue in the Del3-Mpl extracellular domain strongly suggests that *del3-mpl* is constitutively activated by disulfide-linked homodimerization.

The extracellular domain of the mature form of the Env-Mpl protein is devoid of cysteine residues. However, we wondered whether the signal peptide could remain uncleaved in a fraction of Env-Mpl proteins, thus allowing conservation of cysteine 3 and disulfide-linked bridges in these molecules. We assayed Env-Mpl-Tag protein expressed at a high level in one FDC-P1 clone for its ability to form disulfide-linked bonds as described above. We found that the migration profiles of this wild-type protein were identical under reducing and nonreducing conditions and that it migrated as a major species with an apparent molecular mass of 29 kDa, which corresponds to the expected size for a monomer (Fig. 5B). In addition, a smear of proteins with higher molecular masses (30 to 35 kDa) which might represent various glycosylated forms of Env-Mpl protein was observed. This indicated that the Env-Mpl signal peptide is fully cleaved and suggests that covalent dimerization is not involved in *env-mpl* activation.

DISCUSSION

The *env-mpl* oncogene has lost most of the region encoding the cytokine receptor extracellular domain and presumably its ligand binding domain and transmits a mitogenic signal to hematopoietic cells constitutively. Since no mutation occurred in the v-Mpl transmembrane and cytoplasmic regions (29, 32), v-mpl constitutive activation likely results from rearrangement that occurred in the extracellular-domain-encoding portion of the Env-Mpl gene. Since 179 amino acids from the central part of the gp70 are deleted in the protein encoded by the *env-mpl* oncogene, we wondered whether this Env sequence rearrangement had any role in *env-mpl* constitutive activation. We show here that the virus SUPMPLV, in which the F-MuLV Env amino acids 65 to 243 were reinserted, induced in vivo the same pathology as MPLV. However, the latency of the disease was 4 to 6 weeks longer, probably as a result of a less efficient

FIG. 5. Del3-Mpl and wild-type Env-Mpl protein analyses under reducing and nonreducing conditions. (A) Nonidet P-40 lysates of 20×10^6 parental FDC-P1 cells (control) and 20×10^6 *del3-mpl-tag* cells were immunoprecipitated with the P5D4 MAb, separated by reducing and nonreducing gel electrophoresis, and assayed by immunoblotting with the P5D4 MAb. (B) As the expected size for a dimer of Env-Mpl protein is close to the size of immunoglobulin heavy chain (IgH), Western blotting of the Env-Mpl protein was performed from total lysate: lysates of 2×10^6 FDC-P1 cells (control) and 2×10^6 *env-mpl-tag* cells were subjected to reducing and nonreducing SDS-12% PAGE and immunoblotted with the P5D4 MAb. IgL, immunoglobulin light chain.

replication of SUPMPLV. Indeed, MPLV and SUPMPLV are defective retroviruses and need helper F-MuLV to replicate. The 280 N-terminal amino acids of the gp70 contain the envelope region responsible for the ecotropic receptor recognition, and Env sequences present in Sup-Mpl had been previously demonstrated to be responsible for viral interference in vitro (11). Such a phenomenon had also been demonstrated in vivo, leading to protection against retroviral diseases after vaccination with attenuated murine leukemia viruses (3). In addition, we found that the *sup-mpl* oncogene was able to confer growth factor independence on FDC-P1 cells (data not shown). In the course of this study, the pathogenic variant DEL3MPLV was generated in vivo following infection with SUPMPLV, and its oncogene *del3-mpl* had all of the mature *env* sequences deleted, thus conferring a selective advantage to this virus. This mutant induced, with the same latency period, the same myeloproliferative disease as the wild-type virus. These data show that the internal deletion of the *env* gene is not required for MPLV pathogenicity but strongly suggest that it favors efficient virus replication.

We next studied the *del3-mpl* oncogene whose product had all of the Env sequences but the first 12 amino acids of the signal peptide deleted. We demonstrated that a cysteine residue at position 3 in the signal sequence is involved in a disulfide-linked bridge and is strictly required for *del3-mpl* constitutive activation. These results suggest that the Del3-Mpl signal peptide, with its hydrophobic core deleted, stays uncleaved, which agrees with previous results showing that hydrophobicity is required in vivo and in vitro for a functional signal sequence (34). However, subcellular localization studies demonstrated that Del3-Mpl protein is associated with the cellular membrane fraction (data not shown). It is also known that some cellular proteins devoid of canonical signal sequences are targeted to the plasma membrane through an internal membrane-spanning region (30). Thus, the *del3-mpl* oncogene is activated by disulfide-linked homodimerization. Interestingly, an oncogenic form of another cytokine receptor, the erythropoietin receptor, that results from a point mutation in the receptor extracellular domain converting an arginine to a cysteine and that forms disulfide-linked homodimers has been described (16, 24, 36).

The only cysteine residue of the wild-type Env-Mpl extracellular domain is located in the Env signal peptide. Since the entire signal sequence was present in *env-mpl*, we hypothesized that it was cleaved during processing of the Env-Mpl protein. Using confocal microscopy techniques with an anti-Tag MAb (P5D4), we found that the protein Env-Mpl was localized exclusively at the plasma membrane, suggesting that the signal sequence was fully cleaved (data not shown). Moreover, Western blotting of the protein following electrophoresis under nonreducing conditions did not show disulfide-linked homodimers. This suggests that the wild-type *env-mpl* is activated by a mechanism different from covalent homodimerization. Activation as a result of dimerization is a common feature of many growth factor receptors. Unlike most tyrosine kinase receptors, which are composed of a single polypeptide chain that homodimerizes after ligand binding (22), cytokine receptors often have a more complex structure (14, 17, 18, 21, 25, 26, 31). However, ligand-induced dimerization of receptor components has been postulated to be a general first step in their signalling process (13). This has been demonstrated for a number of cytokine receptors. The simplest examples are the granulocyte colony-stimulating factor receptor (9) and the growth hormone receptor (6), whose respective ligands induce homodimerization. In the case of receptor complexes sharing gp130 (receptors for IL-6, IL-11, LIF, OM, and CNTF), li-

gands were shown to induce either homo- or heterodimerization of gp130 (4). IL-6 triggers both a specific disulfide-linked homodimerization of gp130 (19) and a noncovalent association of IL-6 receptor alpha chain with gp130. However, although the sole extracellular domain of IL-6 receptor alpha chain and specific amino acids in this region were shown to be strictly required for this association (35, 40), nothing is known about the mechanism of ligand-induced heterodimerization. We suggest that *env-mpl* is constitutively activated by dimerization involving a noncovalent interaction. Mature Env sequences that, in addition to the 18 N-terminal amino acids of v-Mpl, were deleted in DEL3MPLV could provide this dimerization. Indeed, the soluble extracellular domain of the envelope glycoprotein of the Rous sarcoma virus had been demonstrated to form oligomers in vitro (8, 10). We are currently testing this hypothesis.

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