Characterization of the Gene Encoding the Human LW Blood Group Protein in LW\(^+\) and LW\(^-\) Phenotypes

By P. Hermant, P.Y. Le Pennec, P. Rouger, J.-P. Cartron, and P. Bailly

The LW blood group is carried by a 42-kD glycoprotein that belongs to the family of intercellular adhesion molecules. The LW gene is organized into three exons spanning an HindIII fragment of approximately 2.65 kb. The exon/intron architecture correlates to the structural domains of the protein and resembles that of other Ig superfamily members except that the signal peptide and the first Ig-like domain are encoded by the first exon. The 5′UT region (nucleotides -289 to +9) includes potential binding sites for various transcription factors (Ets, CACC, SP1, GATA-1, AP2) and exhibited a significant transcriptional activity after transfection in the erythroleukemic K562 cells. No obvious abnormality of the LW gene, including the 5′UT region, has been detected by sequencing polymerase chain reaction–amplified genomic DNA from Rh\(^+\) or Rh\(^-\) donors and from an Rh\(^{low}\) variant that lacks the Rh and LW proteins on red blood cells. However, a deletion of 10 bp in exon 1 of the LW gene was identified in the genome of an LW(a⁻ b⁻) individual (Big) deficient for LW antigens but carrying a normal Rh phenotype. The 10-bp deletion generates a premature stop codon and encodes a truncated protein without transmembrane and cytoplasmic domain. No detectable abnormality of the LW gene or transcript could be detected in another LW(a⁻ b⁻) individual (Nic), suggesting the heterogeneity of these phenotypes.

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MATERIALS AND METHODS

Reagents and blood samples. Restriction endonucleases, modifying enzymes, and pUC vectors came from New England Biolabs (Hitchin, Hertfordshire, UK). The Thermus aquaticus polymerase (Taq polymerase) was from Perkin Elmer Cetus (Norwalk, CT). The Sequenase kit was from US Biochemical Corp (Cleveland, OH) and radiolabeled nucleotides were purchased from Amersham (Bucks, UK). Blood samples from common LW and Rh phenotypes came from the Institut National de la Transfusion Sanguine (Paris, France). Samples of Rh\(^{low}\) of the amorph type (D.A.A.) was from Dr C. Perez-Perez (Linares, Spain) and the LW(a⁻ b⁻) blood sample (Big) was kindly provided by Kathy Burnie (Hematology University Hospital, Ontario, Canada). The second LW(a⁻ b⁻) sample (Nic) was discovered recently in a 92-year-old male patient who developed a potent anti-LW\(^{ab}\) antibody.

Isolation of human LW gene. Approximately 2.5 \(\times\) 10\(^7\) phages from a human genomic library (Clontech Laboratories, Inc, Palo Alto, CA) were plated and hybridized under standard procedures with a 32P-labeled full-length LW cDNA probe using a random primed DNA labeling Kit (Boehringer-Mannheim, Mannheim, Germany). One positive clone was isolated and analyzed.

The LW promoter (promoter of the chloramphenicol acetyltransferase (CAT) assays. The SP-A primer (nucleotides [nt] -289 to -268) and AS-A primer (nt 12 to +9) (see Fig 2) were used to polymerase chain reaction (PCR) amplify the 5′ flanking region of the LW gene from a LW(a⁻ b⁻) sample. The PCR product was controlled by sequencing and inserted into the plasmid pHLCAT3\(^{17}\) to generate the LW(-289, +9)-CAT construct. The pHLCAT2 plasmid, containing the ubiquitous promoter of the Herpes simplex thymidine kinase gene\(^{15}\) and the promoterless plasmid pHLCAT3 were used as positive and negative controls, respectively. In each experiment 10 µg of recombinant CAT construct was cotransfected with 2 µg of the RSV-Luc plasmid, carrying the firefly luciferase gene in front of the RSV promoter, into K562 and HeLa cells using electroporation (Bio-Rad Gene Pulser, Hercules, CA) and cationic liposomes (1 mg/mL) according to the guidelines of the manufacturer (GIBCO-BRL, Gaithersburg, MD), respectively. For CAT assays, protein amounts in individual extracts 48 hours after transfection were normalized to luciferase activity and used as described by Gorman et al.\(^{16}\) Experiments were performed twice with two independent plasmid prepara-

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Identification of consensus motifs for potential binding of transcription factors. Four hundred four nt upstream of the transcription initiation site have been sequenced and analyzed to identify putative cis-acting regulatory sequence (Fig 2). There was no typical TATA box in this region, but an inverted CAAT box (ATTG) was present at nt -292. Several potential binding sites for transcription factors were identified in the reverse orientation such as an AP-2 motif (G/GC/CG/C NT/GGGG), a GATA-1 motif (C/TTATC T/A), and the Ets motif (A/GC T/ATCCT/GG/C) at nt -22, -51, and -97, respectively. A reverse CACC motif that could bind a CACC or an SP-1 factor was located at nt -86. In addition, a SP-1 binding site (G/TG/AGGGC/AG/AG/AG/ T) was also identified at nt -72. All these motifs are consensus except the Ets element, which differs by the 5' first nucleotide.

CAT activity of the 5' flanking sequence of the LW gene. The ability of the LW gene region upstream of the first exon to support transcription was tested with the bacterial CAT gene in transient expression assays. Accordingly, a PCR fragment (nt -289 to +9) amplified between SP-A and AS-A primers (see Fig 2) was cloned at the 5' side of the CAT gene in the plasmid pBLCAT3. As negative and positive controls, the promoterless pBLCAT3 plasmid and the pBLCAT2 plasmid containing the ubiquitous promoter of the Herpes simplex thymidine kinase gene were used, respectively. After 48 hours, the LW (-289, +9)-CAT construct showed 225% of CAT activity in erythroleukemic cell line K562 compared with the positive control (Fig 3). In the nonhematopoietic cell line Hela, the same construct showed only 56% of activity (data not shown). These results indicated that the -289 LW gene region, upstream of the first exon, is self-sufficient for transcription in erythroid cells.

Analysis of the LW gene from individuals with different LW and Rh status. The 5' flanking region and the LW gene were PCR-amplified between two sets of primer (SP-A, AS-A and SP-B, AS-B, respectively) from donors with the LW+ and LW- phenotypes and sequenced. All LW+ genomes from RhD+ and RhD- donors exhibited the same LW sequence, including in the 5' UT region. The Rhnull individual (DAA) that lacks LW and Rh antigens also carried an LW gene with a nucleotide sequence identical to that of LW+ individuals. Differences were observed when the two LW(a-b-) individuals (Big and Nic), typed as RhD-, were examined. Sample from Big exhibited a 10-bp deletion (ACCTGCCAGC) in the first exon of the LW allele (see Fig 1), which could be easily detected by PCR amplification between SP-C and AS-C primers (Fig 4A). Because only a 140-bp amplified fragment was amplified from Big instead of the 150-bp fragment seen with the LW+ and Rhnull (DAA) donors, it is obvious that the two LW alleles of Big are affected by the deletion. The 10-bp deletion generated a premature stop codon at the beginning of the exon 2 (see Fig 2) and the resulting mRNA is translated into a truncated protein without transmembrane and cytoplasmic domain. However, in the second LW(a-b-) sample (Nic), there was no deletion and no other abnormality of the two LW alleles could be found. In all cases examined (LW+, LW-, Rhnull), normal LW mRNA was detected following RT-PCR amplification.

Characterization of the human LW gene. Southern blot analysis of human genomic DNAs digested with different restriction endonucleases and probed with the LW-cDNA showed a single-band pattern, suggesting that the LW gene was present as a simple copy in the human genome. The LW gene was finally isolated from an EMBL3 genomic library (5')-CCGGGCCCTGGCTCTCTGGCGC-3', nt -39 to -19) and AS-B (5'-GGCTCAGCCACCATGTATGGCC-3', nt +1672 to +1676). Each cycle consisted of 1 minute of denaturation at 94°C, 1 minute annealing at 60°C, and 2.5 minutes extension at 72°C. After 30 cycles, the 1.2-kb amplified product was ligated in pUC vector and sequenced. For reverse transcription (RT)-PCR analysis of the LW transcripts, total RNAs from whole-blood samples were extracted by the acid-phenol-guanidium method and were reverse transcribed using the first-strand cDNA synthesis kit (Pharmacia, Uppsala, Sweden). The fragment (0.8 kb) amplified between primers SP-D (5'-ATGGGGTCTCTGTTCCCTCTGTCG-3', nt +10 to +53) and AS-D (5'-TTACGCCTGGGACTTCAT-3', nt +102 to +1079) was detected on Southern blot with the LW cDNA probe.

Western blot analysis. RBC membrane proteins from donors of known phenotypes were prepared, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by immunoblotting with rabbit polyclonal antibodies raised against synthetic peptides of the NH2-terminal regions of the LWP and Rh proteins (MPC-8), as previously described.
Fig 2. Nucleotide sequence of the LW gene. Nucleotide sequence of individual exons are shown in uppercase letters and flanking intron sequences in lowercase letters. Exon sequences are boxed and protein translation under each codon is given. Full exon sequences are given in ref 6. Nucleotide positions (left side) are relative to the transcription initiation and not to the translation initiation site as in ref 6. In the 5′-flanking region, potential binding sites for Ets, CACC, SP-1, GATA-1, and AP-2 factors are shown in boldface characters. The 10-bp deletion (nt 355 to 364) in exon 1 of the LW(a- b-) sample Big is indicated by asterisks (*1 and the premature stop codon generated is shown at the beginning of exon 2. The inverted CAAT box and the polyadenylation consensus sequence AATAAA are underlined by double horizontal lines. The SP-A and AS-A primers used for PCR amplification of the promoter region (see Materials and Methods) are indicated. The genomic sequence has been submitted to the EMBL under the accession number X93093.
the LW cDNA clone IV previously identified resulted from the unsplicing of intron 2.

Examination of the sequence immediately upstream of the transcription initiation site showed an inverted CAAT box at position −292 (normally expected at position −70 to −80) but failed to reveal a TATA box. Instead, an inverted consensus sequence for the binding of the GATA-1 transcription factor was identified close to the transcription start site at position −51, as described in several erythroid genes. In addition, binding sites for SP-1, CACC, and Ets are present in close proximity of the GATA-1 motif in the LW gene, as found in regulatory regions of erythroid and megakaryocytic genes. However, although the LW gene is expressed in erythroid tissues, there is presently no indication for megakaryocytic expression.

Sequence comparison of the LW gene from RhD+ and RhD− individuals showed no difference in the region examined (nt −289 to +1,184), offering no explanation for the higher level of LW expression in D+ than D− phenotypes. Further studies should clarify if transcription/translation efficiency or mRNA stability are important factors, although another explanation might be that the RhD polypeptides facilitate LW transport to the cell surface, like glycoporphin A.

Fig 3. Transient expression of the CAT gene directed by constructs containing the 5′ flanking region of the LW gene. K562 and HeLa cells were transfected with the recombinant plasmids LW(−289, +9)CAT, pBLCAT2 and with the promoterless plasmid pBLCAT3 as negative controls. CAT assays were performed 48 hours later, and the products were separated by thin-layer chromatography and autoradiographed overnight. The CAT activity is given as a percentage of the pBLCAT2-transfected cells.

Fig 4. Genomic PCR amplification, RT-PCR transcripts, and protein analysis of LW+ and LW− donors. (A) PCR detection of the 10-bp deletion in the genomic DNA from the LW(−−) donor Big. Primers SP-C (5′-CTCCGGCCACCACCGCGTGGCAGCCGCCGCG-3′, nt +250 to +273) and AS-C (5′-GGCGGTGTCCGGAGGTGCCCAGCG-3′, nt +399 to +425) were used in 50-μL reaction mixtures containing 200 ng of leukocyte DNA (denaturation 1 minute at 94°C, annealing 40 seconds at 60°C, and extension 30 seconds at 72°C). After 30 cycles, the PCR products were resolved on 12% polyacrylamide gel and visualized under UV light after staining with ethidium bromide. A 140-bp instead of a 150-bp expected fragment was amplified from Big. (B) Autoradiogram of the Southern analysis of cDNA reverse-transcribed from LW RNAs by RT-PCR and detected with the LW cDNA probe. (C) Immunostaining of RBC membrane LW and Rh proteins using rabbit antibodies directed against the Rh protein (MPC8, 1:4,000) and the N terminus of the LW protein (1:1,000). Bound antibodies were detected by an alkaline phosphatase-conjugated substrate kit (BioRad). Arrows on the right side indicate the size of Rh and LW proteins.
or glycophophin B enhance the cell-surface expression of the
anion transporter band 326 or the Rh-associated glycoprotein
Rh50,31 respectively.

The LW gene status was next examined in rare individuals
of the LW(a− b−) phenotype that lack LW antigens and LW
protein expression on RBCs. Some of these donors express
normal Rh antigens but others also lack Rh and Rh-associated
antigens and proteins and belong to the group of Rhnull
individuals.32 First, we found that our Rhnull donor (DAA)
of the amorphic type had no detectable LW gene sequence
abnormality (no deletion, mutation, or splice site alteration).
In addition, the LW transcript was indistinguishable from
that of LW+ controls. Together with previous RH gene studies,
these findings support the view that LW and Rh pro-
teins, as well as other Rh-associated membrane proteins,
form a noncovalent complex in the cell membrane that is
not transported efficiently to the cell surface when Rh pro-
teins are lacking.32 At which level and how this complex
assembles in the cell is presently unknown. Next, we exam-
inied two unrelated LW(a− b−) individuals (Big and Nic)
that had a normal phenotypic expression of Rh antigens, as
seen by LW and Rh typing and Western blot analysis (Fig
4). In addition, these cells exhibited a normal phenotypic
expression of the Rh50 glycoprotein and CD47 (our unpub-
lished results, January 1995), which are major membrane
proteins present in the Rh membrane complex.31 We found
Big to be homozygous for a 10-bp deleted LW allele encod-
ing a truncated protein without transmembrane and cyto-
plasmic domains. Most likely, this truncated protein is either
secreted or rapidly degraded, thus explaining the absence of
the LW protein in the RBC membrane (Fig 4). In contrast,
there was no detectable alteration in the LW gene and its
transcript from the second LW(a− b−) sample (Nic). Be-
cause no family study of this patient was available, it cannot
be formerly excluded that the loss of LW antigens and anti-
LW production is transient, as already described in some
cases of pregnancies or immunologic disorders.1 However,
this is unlikely because Nic suffers no such syndrome and
was hospitalized for the removal of a hernia. Therefore,
the reason for the absence of LW protein in this patient remains
obscure and suggests that the LW(a− b−) condition is het-
erogeneous.

In summary, we have clarified the LW gene structure in
individuals that exhibit the LW+ and LW− phenotypes, but
major questions concerning the modulation of LW expres-
sion and the biosynthesis of the Rh membrane complex32
remain unresolved. In addition, future studies should better
delineate whether the structural similarity of LW with inter-
cellular adhesion molecules play any significant biologic
role.

ACKNOWLEDGMENT

We thank Kathy Burnie (Hematology University Hospital, On-
tario, Canada) for the gift of the LW(a− b−) blood sample (Big)
and Dr C. Perez (Linarex, Spain) for the Rhnull blood sample (DAA).
We thank also Martine Huet and Nicole Lucien (Institut National
de la Transfusion Sanguine, Paris, France) for technical assistance.

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