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Selection of the Alternative Exon 1 from the cd5 Gene Down-Regulates Membrane Level of the Protein in B Lymphocytes

Soizic Garaud,* Christelle Le Dantec,* Christian Berthou,† Peter M. Lydyard,‡ Pierre Youinou,²* and Yves Renaudineau*

The human cd5 gene has two alternative exons 1: exon 1A (E1A) which encodes the full-length (FL) CD5 protein and exon 1B (E1B) which encodes a truncated (TR) isoform. The FL variant of CD5 protein is translocated to the plasma membrane, while its TR variant is retained in the cytoplasm. Because there is an inverse relationship between the levels of FL-CD5 and TR-CD5 in B cells, we have addressed the issue of how the selection of exon 1 is determined. In leukemic B cells, DNA methyltransferase (DNMT1)-induced methylation of E1B prevents its transcription. Furthermore, the level of mRNA for DNMT1 correlates inversely with that of mRNA for CD5-E1B. However, suppression of E1B transcription is incomplete, and some molecules of TR-CD5 continue to be synthesized. Bortezomib-induced inhibition of the proteasome establishes that these TR-CD5 molecules are cleared through the ubiquitin-proteasome pathway. Transfection of CD5 mutants into COS-1 cells locates the ubiquitin-binding site at the second destruction box of the extracellular region of CD5. Activation of the B cells by anti-IgM, Staphylococcus aureus Cowan I (SAC), or PMA up-regulates DNMT1, and thereby CD5-E1A mRNA at the expense of CD5-E1B mRNA. Aberrant synthesis of TR-CD5 is thus offset by balanced degradation of excessive protein. Dysregulation of these mechanisms reduces the expression level of membrane CD5, and thereby diminishes the threshold of the response by cells expressing CD5. The Journal of Immunology, 2008, 181: 2010–2018.

The T lymphocyte membrane protein CD5, which has three scavenger cysteine-rich extracellular domains (1), is also expressed by B lymphocytes. The latter cells have been classified according to their expression of CD5 into a minor population of B1 cells with CD5, and a major population of B2 cells without CD5 (2). The B1 cell population is further divided into B1a cells which express surface CD5, and B1b cells which do not. They share other characteristics of B1a cells, including a reduction in the expression level of membrane CD5 (3). Given the key role that CD5 plays in B1 cell physiology, its expression is tightly regulated. So far, two mechanisms have been documented: shedding (4) and internalization (5) of the molecule. A third mechanism involving regulation of gene transcription is rendered possible by the discovery of an additional exon 1 (6). This exon has arisen from the integration of a human endogenous retrovirus (HERV)³ into chromosome 11 of primates, and is, hence, absent in the equivalent mouse gene (7). The newly identified exon 1 has been designated exon 1B (E1B), while the documented exon 1 has been renamed exon 1A (E1A). Remarkably, CD5-E1B-containing transcripts are restricted to B cells, whereas their level correlates inversely with the density of CD5 expression on the cell membrane. Full-length (FL)-CD5 proteins encoded by E1A-containing transcripts translocate to the plasma membrane, but not truncated (TR)-CD5 proteins encoded by E1B-containing transcripts. In these transcripts, the E1A is spliced out, and initiation of the protein synthesis site shifted from ATG1 in E1A to ATG2 in exon 3. Owing to the lack of a leader peptide, the TR-CD5 protein is retained in the cytoplasm. It is, therefore, important how exon 1 is selected for transcription.

The molecular mechanism involved in determining which exon 1 variant is transcribed is currently unknown. One way to silence gene expression is the transfer of methyl groups to CpG motifs within the promoter region of the gene by DNA methyltransferases (DNMTs). Of these, DNMT1 is a maintenance methylase (9). Although the pattern of gene methylation is established early in ontogeny and maintained by DNMT1, such pattern can be reversible (10). For abnormally structured proteins, their regulation occurs through degradation through the ubiquitin-proteasome pathway. This proposition is supported by three observations. First, is the increased activity of the Cbl-ubiquitin ligase in chronic lymphocytic lymphoma (CLL) B cells, which number a proteins including CD5 need regulation (11). Second, is the evidence for a Cbl-binding site in the cytoplasmic domain of CD5 (12). Third, is the availability of two destruction boxes (D boxes) and three phosphorylation sites as ubiquitin-binding sites (UBS) along the extracellular region of CD5.

Because CLL B cells hypermethylate CpG islands (13), and display high levels of cd5-E1A-encoded FL-CD5 (14), these B cells are ideally suited to explore the existence of such regulation.
Their analysis detailed in this study reveal two mechanisms involved in the regulation of distribution of FL- and TR-CD5 isoforms. The first involves blockade of CD5-E1B transcription by DNMTs that favors transcription of a CD5-E1B-containing isoform as a default mechanism. The second involves regulation by ubiquitination of a CD5-E1B-derived TR-CD5 isoform and degradation by the proteasome.

Materials and Methods

Patients and controls

Twenty patients with untreated CLL (15) were recruited for the study, but not all patients participated in all experiments described in this report. Blood from seven healthy volunteers and tonsils from four tonsillectomies were also collected. Informed consent was obtained from the patients or their guardians, and the study protocol was approved by the Institutional Review Board at Brest University.

Cell preparation

PBMCs were isolated by centrifugation on Ficoll-Hypaque. Unless otherwise specified, mAbs were obtained from Beckman Coulter. Following incubation of the PBMCs with SRBCs, those T cells that did not rosette with SRBCs were separated by incubating the remaining PBMCs with anti-CD3, -CD4, -CD8, -CD14, -CD16, -CD19, -CD56, and -CD64 mAbs. Then, B lymphocytes were negatively enriched by incubating the cell suspension with sheep anti-mouse Ig-coated beads (Miltenyi Biotec). In selected experiments, T cells were positively enriched by incubation with anti-CD3, -CD4, -CD8, -CD14, -CD16, -CD19, -CD20, and -CD56 mAbs. The cycle was restarted by reintroduction of serum, and then cell cultures were maintained in the same medium with 1 mM sodium pyruvate, and COS-1 cells cultured in DMEM.

DNMTs were supplied by Prof. A. Dalloul (University of Nancy, Nancy, France). These cells contain transcripts for CD5-E1B but not CD5-E1A (6, 16). Daudi B cells, which are negative for surface CD5 but transfected with cd5-E1A to induce membrane CD5 expression of CD5, were supplied by Prof. A. Dalloul (University of Nancy, Nancy, France) (17, 18). Jurkat T cells and COS-1 cells were purchased from the American Type Culture Collection.

Cell culture

B lymphocytes were cultured in RPMI 1640 supplemented with 10% FCS, 2 mM-glutamine, and antibiotics. Transfected CD5 B cells were maintained in the same medium with 1 mM sodium pyruvate, and COS-1 cells cultured in DMEM.

DNMTs were inhibited by a 48-h incubation with either 100 μM of the competitive inhibitor probenecid (PcA) or 50 μM of the irreversible inhibitor 5-azacytidine (aza). Based on the dependence of DNMT expression on MAPK activation (19), 50 μM of the ras-signal blocker PD98059 (Merck) was also used.

To block the proteasome, B cells were incubated with 10 nM bortezomib (BTZ) for 24 h. For arresting the cell cycle in the G1 phase, the lymphocytes were incubated with 400 μM t-mimosine (Sigma-Aldrich) for 48 h. For keeping the cells in the G1 phase, they were deprived of serum. The cycle was restarted by reinduction of serum, and then cell aliquots were harvested at several time points for RT-PCR.

Finally, B lymphocytes were activated by 10 μg/ml anti-IgM mAb-coated Sepharose beads (Bio-Rad) in the presence of 10 U/ml IL-2, while Staphylococcus aureus Cowan I (SAC) and PMA-induced activations served as positive controls (6). FITC-anti-CD69, FITC-anti-CD80, and PE-anti-CD86 staining confirmed that the cells were activated.

FACS analyses

Anti-CD5 mAbs can be classified into two groups based on the epitope they recognize (20). The first group (exemplified by “UCHT2”) recognize both the FL and the TR forms of CD5, and the second (exemplified by...
“Leu1”) recognize only the FL form of CD5 (6). The epitope targeted by Leu1 is assigned to the 11 N-terminal residues of CD5, which are missing from TR-CD5 protein. Leu1 was, therefore, selected to identify FL-CD5.

With regard to TR-CD5, we used UCHT2 (BD Pharmingen), and the anti-CD5 54/F6 mAb (DakoCytomation) raised against a peptide from the intracellular domain of CD5 (21). PBMCs were seeded at 5 x 10^5 cells/tube and incubated with FITC-anti-CD19 and Leu1 PE-anti-CD5 for 30 min at 4°C. After three washes, the cells were analyzed on an EPICS-XL FACS machine (Beckman Coulter) and their mean fluorescence intensity (MFI) was expressed relative to that of the isotype control.

To stain cytoplasmic CD5, the cells were permeabilized with 0.5% saponin (Sigma-Aldrich), incubated with UCHT2 anti-CD5, or 54/F6 mAb, followed by biotinylated anti-mouse Ig, and then FITC-streptavidin (Amersham). DNMT1 was detected by intracellular staining after permeabilization of the lymphocytes with 70% methanol.

Conventional and quantitative RT-PCR

Total mRNA was extracted using the RNAble kit (Eurobio). One microgram of the mRNA was reverse-transcribed to generate cDNA using random hexamers. The PCR amplification of CD5 and DNMT cDNA involved an initial denaturation cycle at 94°C for 5 min, followed by 40 cycles of 94°C for 1 min for denaturation, 65°C for 40 s for annealing, and 72°C for 1 min for extension, followed by a final 10-min extension at 72°C. The PCR protocol for GAPDH mRNA was the same, except that there were only 35 annealing cycles. In the end, cDNA was analyzed on a 2% agarose gel and DNA was visualized with ethidium bromide.

Quantitative RT-PCR was conducted in triplicate tubes of 20-μl mixtures containing 50 ng of cDNA, 500 nM of each primer (Table I), and 1× SYBR green PCR master mix (Applied Biosystems). Included in each assay were the reaction mixture with no template as a negative control, and

FIGURE 1. Cytoplasmic TR-CD5 affects the membrane expression of FL-CD5. CLL CD5^- B cells from four patients in A, and three batches of CD5^- Daudi B cells in B (filled curves) were transfected with control GFP (upper panels), or with fusion cd5-E1B-GFP constructs (lower panels). The cells were stained with PE-anti-CD5, and CD5 surface expression in GFP-positive cells was compared with GFP-negative cells (open curves) and isotype controls (dotted lines).

A

B

CDL B cells

CD5+ Daudi B cells

GFP

CD5

GFP

CD5

FIGURE 2. DNMTs control the transcription of E1B. A. Left, Inhibition of DNMTs with PcA or aza, and blockade of MAPKs (on which expression of DNMT1 is dependent) with PD98059, decreases the ratio of CD5-E1A to CD5-E1B transcripts in B cells from eight CLL patients and four normal controls. Right, The membrane MFI of CD5 is also reduced in a dose-dependent manner. B. Intracellular staining of DNMT1 in B cells before (top) and after (bottom) treatment with PD98059 (thick lines), compared with isotype control (thin lines). C. The expression of DNMTs was also dependent on the cell cycle. This was blocked by l-mimosine (left), or by serum deprivation (right). The transcripts for DNMT1, cd5-E1B, cd5-E1A, and GAPDH were assessed by RT-PCR. Serum was then reintroduced, and the transcription was assessed. D. Methylation status of the cd5-E1B promoter. Left, Following digestion with methylation-insensitive MspI or methylation-sensitive HpaII. Genomic DNAs were amplified, and 1436-bp amplicons were shown to be methylated on their five 5'--CCGG-3' restriction sites targeted by the enzymes (arrowed in E). Right, DNMT inhibitors demethylated at least one of these five restriction sites. E, Bisulfite-treated genomic DNAs were amplified to identify which ones of the 26 CpGs were demethylated. The splice donor (SD) and the CpG polymorphism (★) are indicated.
Table II. Relationship between the transcripts for DNMT1 and those for E1B- and E1A-containing CD5 in subpopulations of tonsillar B cells

<table>
<thead>
<tr>
<th>Subpopulations</th>
<th>DNMT1</th>
<th>E1A</th>
<th>E1B</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1a</td>
<td>18.9 ± 4.6</td>
<td>13.7 ± 2.5</td>
<td>5.2 ± 2.0</td>
</tr>
<tr>
<td>B1b</td>
<td>20.4 ± 12.6</td>
<td>5.3 ± 2.6</td>
<td>11.2 ± 6.3</td>
</tr>
<tr>
<td>B2</td>
<td>88.9 ± 8.6</td>
<td>1.8 ± 1.9</td>
<td>0.8 ± 1.3</td>
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* Transcripts were enumerated by quantitative PCR and the results were normalized to 18S rRNA values (×10^6). Data are from four tonsillar B cell preparations (means ± SD).

the mixture with 18S rRNA as a positive control. The number of mRNA copies was deduced from the threshold cycle numbers and standard curves normalized to 18S rRNA values.

**Methylation-specific PCR**

The assay is based on the inability of selected enzymes to digest a methylated 5'-CCmGG-3' site (22). B cell genomic DNAs of six CLL patients and four healthy controls were purified using the QIAmp 96 DNA kit (Qiagen), and digested with 20 U of the methylation-sensitive enzyme HpaII, or the methylation-insensitive enzyme MspI (Invitrogen) for 3 h at 37°C. The forward primer was designed to anneal upstream of 5'LTR and the reverse primer to the E1B (6). The 1436-bp product obtained after 35 cycles at 56°C was resolved and visualized as above.

**Bisulfite sequencing**

To analyze DNA methylation, genomic DNA was isolated, and nonmethylated cytosines converted to uridines with bisulfite treatment using the EZ-DNA Methylation-Gold kit (Zymo). The 5'-CCmGG-3' site (22). B cell genomic DNAs of six CLL patients and four healthy controls were purified using the QIAmp 96 DNA kit (Qiagen), and digested with 20 U of the methylation-sensitive enzyme HpaII, or the methylation-insensitive enzyme MspI (Invitrogen) for 3 h at 37°C. The forward primer was designed to anneal upstream of 5'LTR and the reverse primer to the E1B (6). The 1436-bp product obtained after 35 cycles at 56°C was resolved and visualized as above.

**Production of rCD5 vectors**

The Jurkat T cell line was the source of CD5-E1A transcript, and the 697 bp pre-B cell line was the source of the CD5-E1B transcript. For CD5-E1A, the sense primer was associated with an ATG site adjacent to a Kozak sequence, while the antisense primer was located downstream of the stop codon. Amplification was completed using a mixture of Pwo and Taq polymerases.

The cd5-E1A and cd5-E1B cDNAs were cloned into the pTrueBlue vector (Genomics One). For amplification, a sense primer was placed upstream of the kpnI restriction site, and an antisense primer was designed to overlap with the 3' region of exon 11 and the 5' end of the nearby sequence. In addition to its amplifying effect, this protocol substituted an EcoRI restriction site for the stop codon to permit translation of the 3' sequence of cd5. Once cut, the CD5-E1A fragment was subcloned into the red fluorescent protein (RFP) gene-containing Red2-N1 plasmid, and the CD5-E1B fragment into the GFP gene-containing pGFP-N1 plasmid (BD Clontech).

**Construction of deletion mutants of CD5**

Three deletion mutants were designed: L137Mstart in which the first D box at position 134 was removed, L286Mstop in which the second D box at position 283 was removed, and L403Mstop in which the whole extracellular part of the protein was removed. All mutants were derived from CD5-E1B-GFP by PCR, using ATG-containing sense primers, and the same antisense primer. The constructs were cloned into the pDNR dual, subcloned into the plp CMV vector, using the Cre-recombinase system (both obtained from BD Clontech), and validated by DNA sequencing.

**Directed mutagenesis of CD5**

To identify residues involved in the binding of CD5 to ubiquitin, the cd5-E1B cDNA was mutagenized using the Quick-Change Site-Directed Mutagenesis kit from Stratagene. Point mutations were introduced into the three serine phosphorylation sites (218SSctS222, 258SS259, and 279SSS281), and the second D box (282aRSSL286). Then, CD5-E1B-GFP was used as a template, and the mutated constructs, again, were validated by DNA sequencing.

**Transient transfection**

The cd5-E1B-GFF gene-containing plasmid was transiently up-regulated in CLL CD5+B cells (using a V kit VCA-1003), and in CD5+ Daudi B cells (using the B Cell Nucleofector kit VPA-1003), according to Amaya’s instructions. COS-1 cells were transiently transfected with 1 μg of cd5-E1A-RFP vector (or control RFP), or with 1 μg of cd5-E1B-GFP vector (or control GFP), using the same kit. DMEM medium replaced the OptiMEM medium after a 3-h incubation, and 10 nM BTZ was added. The cells were incubated for a further 24 h, and harvested with 0.25% trypsin. The resulting fluorescence was examined by confocal microscopy and by FACS.

**Statistical analysis**

Results were expressed as arithmetic means ± SD, and compared using the Mann-Whitney U test for unpaired data, and the Wilcoxon’s test for paired data.

**FIGURE 3.** DNMT1s are triggered by BCR engagement. A. Cross-linking the BCR of B cells from six CLL patients raised the ratio of CD5-E1A to CD5-E1B transcripts (left), and the MFI of CD5 (right) in time-dependent manners. B. There was also a time-dependent increase in the level of DNMT1 transcripts. C. Increases in the expression of the DNMT1 protein by anti-IgM, SAC, or PMA. D. These were associated with the CD69, CD80, and CD86 activation markers (dotted lines represent isotype controls).
Results

cd5-E1B lowers membrane CD5 expression levels

To substitute CD5-E1B transcripts for CD5-E1A transcripts, cd5-E1B-GFP (or control GFP) was transfected into four samples of CD5-E1A-positive CLL B lymphocytes and three batches of CD5-E1A-positive Daudi B lymphocytes. The cells were then gated into GFP/H11001 and GFP/H11002 fractions to distinguish cd5-E1B-transfected cells from nontransfected cells. The expression of CD5 was measured by FACS using PE-anti-CD5. After transfection of cd5-E1B-GFP, the level of CD5 expression diminished from 20.6 ± 0.6 to 5.0 ± 0.5 in CLL B cells (compare A1 with AII in Fig. 1A; p < 0.05), and from 48.3 ± 1.3 to 19.3 ± 1.0 in CD5+ Daudi B cells (compare B1 with BII in Fig. 1; p < 0.05). Thus, the results show that enhanced transcription of cd5-E1B lowered membrane CD5 expression levels in B lymphocytes, as we previously observed for cd5-E1B cDNA-transfected Jurkat T cells (6).

DNMTs regulate exon 1 transcription

To test the likelihood that the promoter of cd5-E1B is regulated by methylation, the effects of DNMT inhibitors and MAPK blockers (on which expression of DNMT is dependent: see Ref. 19) were evaluated. In pilot experiments, the ratios of CD5-E1A to CD5-E1B transcripts were measured in the presence of PcA, aza, or PD98059 (Fig. 2A, left), and that of cd5-E1B proportionally up-regulated. Cells were also arrested in the G1 phase by serum deprivation, reducing DNMT transcription so that cd5-E1B was poorly methylated, and, thus, transcribed (Fig. 2C, right). Reintroduction of serum restarted DNMT activity and restored the E1B blockade in a time-dependent manner.

Methylation status of the cd5-E1B promoter

To further verify the importance of DNMTs, methylation of CpGs in the E1B promoter was studied. Following digestion with methylation-insensitive MspI, or with methylation-sensitive HpaII, genomic DNAs were amplified, and the five restriction site-included CpGs were methylated (Fig. 2D, left). DNMT inhibitors
FIGURE 5. Proteasomic degradation of excessive CD5-E1B in COS-1 cells. A, Confocal microscopy of COS-1 cells at constant settings. Each image is representative of many cells from at least three experiments. The cells were transfected with RFP or cd5-E1A-RFP, with GFP or cd5-E1B-GFP, and treated or not with BTZ. GFP alone is shown as a control. B, The MFI of cells transfected with control GFP was assigned a value of 100, and those of cells transfected with cd5-E1B-RFP were expressed as percentages of this reference MFI. C, Expression of CD5 in COS-1 cells transfected with CD5 deletion mutants, and treated or not with BTZ. D, Right, Schematic representation of cd5-E1B and deletion mutants. CD5 molecules comprise three extracellular domains (D), a transmembrane (Tm) region, and a cytoplasmic (Cy) portion. RxxL are D boxes. CD5 deletion mutants (L137Mstart, L286Mstart, and M403Mstart) are named by the start codon and its position in the mature wild-type sequence. Left, FACS analysis of COS-1 cells transfected with GFP, cd5-E1B-GFP construct, or deletion mutants of CD5.

demethylated at least one of them (Fig. 2D, right). Bisulfite sequencing revealed that of a total of 26, demethylated CpGs were located within and upstream of the U3 sequence (white boxes in Fig. 2E). In view of the inverse correlation between transcription of E1B and density of surface CD5, we sorted B1a, B1b, and B2 cells, and estimated transcripts of E1A, E1B, and DNMT1 by quantitative RT-PCR (Table II). Levels of E1A were higher in B1a than in B1b cells, and in B1b than in B2 cells. Conversely, transcripts of E1B were lower in B1a than in B1b cells, in concordance with decreasing amounts of DNMT1.

In summary, levels of DNMT1 transcripts correlated inversely with those of CD5-E1B transcripts. Although somewhat variable, a low transcription of DNMT was associated with a high transcription of cd5-E1B in B1a and B1b cells, and a high transcription of DNMT with a low transcription of cd5-E1B in B2 cells.

DNMTs are switched on following B cell activation

Treatment of B2 cells with anti-Ig induces CD5 surface expression (24). To determine whether DNMTs interfere with this process, we examined the effects of B cell activation on DNMT expression in cells from six CLL patients. The redistribution of transcripts into CD5-E1A and CD5-E1B was also investigated.

Engagement of the BCR with anti-IgM increased the ratio of E1A- to E1B-containing transcripts (Fig. 3A, left; p < 0.05 at 24 and 48 h) and enhanced the density of membrane CD5 protein (Fig. 3A, right; NS at 24 h, and p < 0.05 at 48 h). There was also an increase in the level of DNMT1 mRNA (Fig. 3B; p < 0.05 at 24 and 48 h). These findings imply that activation of B cells by anti-Ig, SAC, or PMA, induces transcription of DNMT1 and eventually up-regulated CD5 expression. Interestingly, the increase in the DNMT1 levels was associated with increases in the CD69, CD80, and CD86 activation marker levels (Fig. 3C).

Expression level of FL-CD5 is influenced by degradation of TR-CD5

When the repression of E1B-containing cd5 transcription is incomplete, limited CD5-E1B transcripts are generated. One consequence of this leak is that the level of CD5-E1B is then likely to be regulated at the RNA or TR-CD5 protein level by proteolysis. To determine the likelihood of such scenarios, degradation of ub proteins in the proteasome was blocked with BTZ in B lymphocytes from seven patients with CLL. Controls for CD5-E1A were CD5° Daudi B cells, while those for CD5-E1B were 697 pre-B cells. MFI for FL-CD5 expression diminished from 23.1 ± 0.2 to 10.9 ± 0.1 on the membrane (bold histogram in the first panel of the first row of Fig. 4A; p < 0.05), while that of TR-CD5 increased from 22.8 ± 0.4 to 27.0 ± 1.5 in the cytoplasm (bold histogram in the first panel of the second row; p < 0.05). Similar changes to the level of FL-CD5 (second, third, and fourth panels of the first row) and TR-CD5 (second, third, and fourth panels of the second row) were not seen. Neither were changes to the membrane level of CD3, CD20, and CD72 (first row of Fig. 4B). There were, however, modest effects on CD18, CD22, and CD23 (second row of Fig. 4B), and marked effects on CD27, CD40, and CD45RO (third row of Fig. 4B), indicating that this phenomenon was not restricted to CD5.

Next, we examined whether the FL-CD5 protein was protected when TR-CD5 was degraded in the proteasome. Two sets of three aliquots of COS-1 cells were analyzed. The first set was kept for analysis of FL-CD5: its aliquot 1 was transfected with cd5-E1A-RFP, its aliquot 2 was similarly transfected but treated with BTZ, while its aliquot 3 was transfected with control RFP. The second set was used for analysis of TR-CD5: its aliquot 1 was transfected with cd5-E1B-GFP, its aliquot 2 was similarly transfected but
treated with BTZ, while its aliquot 3 was transfected with control GFP.

FACS analyses revealed that BTZ had no effects on membrane FL-CD5 in cd5-E1A-RFP-transfected cells (data not shown). In contrast, the MFI for cytoplasmic TR-CD5 was enhanced from 37.4 ± 0.6 to 103.2 ± 5.5 in cd5-E1B-GFP-transfected cells (p < 0.01, five experiments). These results were confirmed using confocal microscopy in which at least 50 cells were examined (representative prints are shown in Fig. 5A). To minimize the effects of variations, the MFI for GFP-transfected cells was assigned a value of 100 and that of cd5-E1B-transfected cells was expressed as percentages of this MFI. Transfected cells showed a reduction from 100 to 11.7 ± 1.1 in the absence of BTZ and to 27.8 ± 0.2% in its presence (Fig. 5B). In brief, TR-CD5 disappeared at the proteasome normally, but accumulated at the proteasome in the presence of BTZ.

Localization of UBS within the CD5 protein

The extracellular region CD5 contains two D boxes and three serine phosphorylation sites that could potentially be involved in ub transfer

### FIGURE 6

In vitro proteasome degradation of TR-CD5 is reduced significantly by ^282_aRSSL^286_to^282_VDSSV^286 substitutions. A, Confocal microscopy analysis of transfected COS-1 cells. B, Constructs made by directed mutagenesis. C, FACS analysis of COS-1 cells expressing fusion cd5-E1B-GFP, or mutants fused with GFP. The values for each experiment are represented as percent fluorescence of the MFI of GFP^+ control cells. Mean ± SD of at least three experiments.

### FIGURE 7

Colocalization of FL-CD5 and TR-CD5. COS-1 cells were transiently transfected with cd5-E1A-RFP (column 1) and with cd5-E1B-GFP (column 2). Overlay of RFP-stained FL-CD5 and GFP-stained TR-CD5 is seen as yellow (column 3), and superimposition suggests intracellular colocalization of both isoforms of CD5. In some experiments, CD5 deletion mutants (L137M in column 4, L286M in column 5, and M403M in column 6) were used, instead of cd5-E1B-GFP. The cells were examined by confocal microscopy, acquired using constant settings. Overlay of the two images shows intracellular colocalization, when the cells are seen as yellow.
of the protein into the proteasome. Pilot experiments with the deletion M403Mstart mutant pointed to the involvement of this region in the degradation of CD5-E1B because CD5 could not be degraded in its absence (Fig. 5, C and D). In contrast, L137Mstart-CD5 was degraded, while L286Mstart-CD5 was not, indicating that the first D box was not involved in CD5 transport to the proteasome. None of the three serine phosphorylation sites were involved in the degradation of CD5 (Fig. 6), because 218SSctSS222→218VDctV222, 258SS259→258VD259, and 279SS281→279VD281 mutants did not influence the fate of CD5.

In contrast, the second D box was involved in the degradation, as suggested by the restraint in the degradation of CD5 following mutation of 282rRSSL286 into 282VDSSV286. However, this mutation was not sufficient to rescue all molecules of TR-CD5 from degradation. Despite this mutation, some molecules remained degradable, and were, thus, preserved by BTZ. Variations in the catalytic rate of TR-CD5 have effects on the membrane FL-CD5.

To summarize the experiments described in this section, the more TR-CD5 degraded in the proteasome, the more FL-CD5 molecules were expressed on the membrane. In contrast, the more TR-CD5 escaped from the proteasome the fewer FL-CD5 molecules were expressed on the membrane.

**TR-CD5 interacts with FL-CD5 intracellularly**

Our finding that CD5-E1B alters the level of membrane expression of CD5 raises the possibility that TR-CD5 interacts with FL-CD5 intracellularly. To address this issue, COS-1 cells were transfected with cd5-E1A-RFP (or with the corresponding empty vector), and cd5-E1B-GFP (or with the corresponding empty vector) simultaneously. Confocal microscopy indicated that RFP-tagged FL-CD5 was translocated to the membrane (Fig. 7, column 1), and the GFP-tagged TR-CD5 was retained in the cytoplasm (Fig. 7, column 2). Expression of both variants partially induced translocation of both proteins in the form of cytoplasmic aggregates. As a consequence, the overlay of RFP-stained FL-CD5 and GFP-stained TR-CD5 is seen as yellow (Fig. 7, column 3). Again, CD5-E1B deletion mutants unraveled the sites used by TR-CD5 to bind to FL-CD5. These appear to be located upstream of amino acid position 404, because constructs L137Mstart and L286Mstart of TR-CD5 were kept associated with FL-CD5 (Fig. 7, columns 4 and 5), whereas constructs M403Mstart were not (Fig. 7, column 6).

**Discussion**

Numerous findings indicate that membrane CD5 expression is tightly regulated. Thus, the density of CD5 on the surface of T cells is 30-fold higher than in B1a cells (25). In T lymphocytes themselves, there is less CD5 on immature than on mature cells (26). Furthermore, there is evidence that the proportion of CD5-expressing B cells declines with age (27), and that the density of CD5 molecules on B cells is reduced by IL-4 which is important for their differentiation (28), but increased by PMA stimulation (29).

Following from the original observations suggesting the relevance of CD5 expression in pathophysiology, increasing data highlight the role CD5 plays in the physiology of B cells. Indeed, CD5 associates with SHP-1 (30) and the BCR (31), and influences proximal signaling in B cells. Furthermore, there is a causal relationship between CD5 expression and transcription of RAGs 1 and 2 (32). CD5 mRNA persists in CD5-negative B1b cells (2), and CD5 expression disappears on EBV transformation (33). Although in mice CD5 expression depends upon the interplay between several mechanisms, it remains unclear whether similar mechanisms operate in humans. However, we have demonstrated that the alternate E1B exon could not be found in mice, whereas both exons 1 can be transcribed in humans. The resultant cd5-E1A-encoded FL-protein is expressed on the membrane while the cd5-E1B-encoded TR protein is retained in the cytoplasm.

To gain insight into mechanisms that ultimately determine which E1A or E1B transcript is expressed, we first examined the factors that determine transcription. On the basis that the E1B sequence is derived from an HERV (7), and that CpG methylation regulates the transcriptional activity of HERVs (8), we presumed that DNMTs were likely to be involved in the regulation of the cd5 gene. The inhibition of DNMTs demethylates it, and thereby increases the number of CD5-E1B transcripts. Thus, TR-CD5 proteins are eventually synthesized at the expense of FL-CD5. As a result, more cytoplasmic TR-CD5 would be associated with less surface FL-CD5. Similar effects were observed following transfection of cd5-E1B into CLL and CD5+ Daudi B cells. Interestingly, the noted decreases in the level of CD5 protein expression were modest (Fig. 1), compared with the transcripts (Fig. 2A). One explanation for this discrepancy could be the relatively slow kinetics of CD5 synthesis that reaches a plateau in 36 h while, for example, that of CD69 takes 12 h (34).

Our results also suggest that transcription of cd5-E1B may not be completely regulated by DNMTs and, therefore, other mechanisms would be involved to remove TR-CD5 protein. Our inhibition experiments with DNMTs suggest that excessive TR-CD5 could be controlled by the ubiquitin-proteasome pathway. The fact that M403Mstart-encoded CD5 could not enter the proteasome indicates that the UBS are located in the extracellular domains. That the L137Mstart, but not the L286Mstart, construct was degraded specifies that the initiation site is somewhere between position 137 and position 286. Alternatively, CD5 might be ubiquitinated via one of the three phosphorylation sites between these positions.

The data, thus, suggest that cytoplasmic TR-CD5 regulates FL-CD5 membrane expression. In CLL B cells (23), DNMTs are continuously generated. However, their synthesis during the G0/G1 phase is down-regulated. Despite the arrest of CLL B cells in the G0 phase, their DNMTs do totally suppress the transcription of CD5-E1B-containing transcripts. As a result, some TR-CD5 molecules are synthesized, and eventually degraded in proteasome (35). This results in the enhancement of membrane CD5 expression (36). There is also evidence to suggest that CD5 is involved in promoting survival. Natural phosphorylation of regulatory tyrosines on CD5 in CLL B cells reflects chronic activation (18), and sustained signaling through the BCR promotes further expression of CD5 (37). Thus, BCR engagement favors the expression of CD5 (38) which provides viability signals for B cells in a subset of CLL patients (39, 40). The prediction from these results is that chronic activation of malignant B cells (17) results in their protection from apoptosis.

Importantly, results generated in this study showed that regulation of membrane CD5 expression by methylation is restricted to B cells, because T cells retain surface CD5 expression in the presence of DNMT inhibitors. Therefore, T cells use other mechanisms to suppress E1B expression. These differences between B and T cells are consistent with the specificity of the role of E1B in B cells. In malignant B lymphocytes (41), transcription of DNMT genes is triggered by activation of STATs. These bind to TT and AA tandem base-containing DNA sequences. It is of note that they are found in the promoter of E1B (7). Furthermore, DNMTs are related to STAT-binding tandem repeats in T cells or EIB-promoting sequences in B cells. Our proposition is supported by two observations: the murine genome interval Slelab encodes STAT3-activating proteins (42), and human CLL B cells contain constitutively activated STATs (43). The role of IL-6 in this activation is being explored (44–46), particularly in view of hypomethylation of DNA in autoreactive T cells from patients with SLE (47) or drug-induced lupus (48).
In conclusion, our studies show that an inverse relationship exists between levels of membrane FL-CD5 and cytoplasmic levels of TR-CD5. This appears to be an important B cell-specific mechanism developed to regulate the threshold of BCR signaling. Thus, hypomethylation becomes an area worthy of further study in B cells from patients with autoimmune diseases and lymphoproliferative disorders.

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References

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