International Immunology, Vol. 13, No. 5, pp. 705–711

Regulation of CD21 expression by DNA methylation and histone deacetylation

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Keywords: B lymphocyte, chromatin, complement receptor II, CR2, differentiation

Abstract

The complement receptor II (CD21) serves as a receptor for the complement component C3d of immune complexes on B lymphocytes. Expression of the CD21 gene is tightly regulated during B lymphocyte differentiation. Only mature B lymphocytes, but not pro-, pre- or plasma B lymphocytes, express CD21. There is evidence that cell type-specific expression is mediated by a silencer element located in the first intron. The CD21 promoter region contains a CpG island adjacent to the ATG start codon. We have analyzed the methylation status of this CpG island in B lymphoid cell lines representing the various differentiation stages of B lymphocyte development and primary lymphocytes. We found that the pro-, pre- and intermediate B lymphocytes contain a methylated CpG island and do not express CD21, whereas CD21-expressing mature B lymphocytes, plasma B lymphocytes and non-lymphoid cells carry a demethylated CD21 CpG island. To analyze whether the lack of CD21 expression in early B lymphocytes is due to inhibition by CpG methylation we have used 5-aza-2′-deoxycytidine to inhibit DNA methyltransferase activity. Treatment of pro-B lymphocytes with the drug resulted in expression of CD21. We have also applied Trichostatin A (TSA), an inhibitor of histone deacetylation, to determine whether the state of histone deacetylation affects the expression of CD21. We found that TSA induces expression of CD21 in early B lymphocytes. Thus CD21 expression is controlled by both methylation of the CD21 CpG island and chromatin modification through histone deacetylation in early B lymphocyte development.

Introduction

The CD21 gene encodes a regulator of complement activation and resides within a gene cluster on human chromosome 1 (1q32) together with DAF and other complement family genes (1,2). The CD21 glycoprotein has a mol. wt of ~145 kDa, and is expressed in B lymphocytes, T lymphocytes, follicular dendritic cells, astrocytes, basophils and on nasopharyngial epithelial cells (3–14). On B lymphocytes CD21 serves as a receptor for the complement fragment C3d bound to immune complexes and is crucial for mounting immune responses to low-affinity antigen (15–19). CD21 is expressed only in mature cells, but not in pro-, pre- or plasma B lymphocytes (20,21). Two regulatory regions have been defined controlling expression of the human CD21 gene, a promoter residing 5′ of the ATG start codon and a silencer located within the first intron (22–24). One element of the CD21 gene promoter is a typical CpG island with stretches of CpG dinucleotides. These CpG dinucleotides are targets of DNA methyltransferases (DNMT) transferring methyl groups to the carbon 5 position of cytosine. Methylation of CpG islands within regulatory elements frequently results in silencing of transcription (25,26). An important mechanism of methylation-dependent gene silencing is the recruitment of methylcytosine-binding proteins (MeCP1 and 2). MeCP2 associates with the co-repressor mSin3A/histone–deacetylase complex through its transcriptional repression domain. This interaction leads to repression of transcription, which can be relieved by Trichostatin A (TSA) (27).

In the immune system regulation of transcription by methylation is a widely used mechanism to control gene expression, and is involved in VDJ and class switch recombination as well as allelic exclusion (28–31).

Here we report that the CpG island in the CD21 promoter is involved in the expression control of the CD21 gene in early B lymphocyte development. We show that the CD21

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Transmitting editor: A. Radbruch *Received* 30 September 2000, *accepted* 14 February 2001

CpG island appears to be methylated in pro- and pre-B lymphocytes, while the CD21 gene promoter in mature B lymphocytes is not methylated. In support of this we found that 5-aza-2′-deoxycytidine (5-aza-C) treatment of early-stage B lymphocytes renders the CD21 promoter CpG island free of methylation and allows CD21 expression. Furthermore, inhibition of histone deacetylation by TSA leads to the expression of CD21 suggesting that both methylation and changes in the chromatin structure are controlling CD21 expression during B lymphocyte differentiation.

Methods

Methylation analysis

Genomic DNA was isolated from cell lines by standard procedures. DNA (14 μg) was digested with 75 U of each of the restriction enzymes *Msp*I, *Hpa*II, *Eco*RI or *Eco*72I (MBI Fermentas, St Leon-Rot, Germany) and resolved on 1% agarose gels in TBE. DNA was transferred using the alkaline transfer protocol (32) onto Gene Screen Plus membranes (NEN, Zaventem, Belgium).

Filters were hybridized with the ^{33}P-labeled insert of p21pro and analyzed by autoradiography (33).

Cloning, library screening and sequencing

Using primers KN-84 (AAG CTT CCA GCC AAA GGC TG) and KN-81 (AGC TCT GAG CCA CTC TTT GG), a 1108-bp fragment of the CD21 promoter region was amplified from the yeast artificial chromosome clone A29D12 (HGMP Resource Center Hinxton, Cambridge, UK). The PCR product was cloned into pCR2.1 (Invitrogen, Groningen, Netherlands) and termed p21pro. A 380-bp *Eco*RI fragment thereof was purified, labeled with ^{33}P (Amersham Pharmacia, Freiburg, Germany) and used as a probe to analyze the methylation status of the CpG island in the CD21 promoter. Plasmid pDAF was cloned from a PCR product amplified with primers KN139 (TTT GGT GCA ACC ATC TCC TTC T) and KN140 (TTC CTC TGC ATT CAG GTG GTG) from cDNA of IM9 cells, and represents part of the DAF gene. A human genomic BAC library (RPCI-11, Resource Center of the German Genome Project, Berlin, Germany) was screened with probes derived from plasmids p21pro and pDAF to find a clone with an insert covering the region between the 3′ end of the DAF gene and CD21. One BAC clone (RPCI-11 289E13) was propagated, shotgun cloned into Topo pCRII blunt and sequenced. A contig of 14 kb was submitted to GenBank (accession no. AF 298224).

Cell lines and tissue culture

The pro-B cell lines RS4;11 (34), Nalm16 (35), KM3 (36) and REH (37), the pre-B cell lines OB5 (38), 697 (39) and Nalm6 (35), the intermediate B cell line 1E8 (38), the mature B cell lines Raji (40), Dakiki (41) and IM9 (42), the plasma B cell lines HS Sultan (43), OPM2 (44), RPMI8226 (45) and U266 (46), the fibroblast cell line K4 (47), the mammary carcinoma cell line MCF-7 (48), the cervical adenocarcinoma cell line HeLa229 (49), and the T cell lines HUT78 (50) and Jurkat (51) were maintained in IMDM (Gibco/BRL, Eggenstein, Germany). The medium was supplemented with 10% fetal bovine serum, 3024 mg/l sodium bicarbonate, 100 U/ml penicillin and 100 μg/ml streptomycin.

For 5-aza-C (Sigma, Munich, Germany) treatment, cells were diluted to 3–4×10^5/ml and allowed to grow overnight. Freshly prepared 5-aza-C was added to a final concentration of 4 μM and the cells were allowed to grow for 72 h before total RNA was prepared using RNAzol B (WAK-Chemie, Bad Soden, Germany). For TSA (Calbiochem, Bad Soden, Germany) treatment cells were diluted to 3–4×10^5/ml and allowed to grow overnight. Freshly prepared TSA was added to a concentration of 300 ng/ml and the cells were allowed to grow for 24 h before total RNA was prepared. Tonsil and blood was obtained from a patient undergoing therapeutically indicated tonsilectomy.

cDNA synthesis and RT-PCR analysis

Quality of the prepared RNA was verified by gel electrophoresis. RNA (5 μg) was used to synthesize cDNA according to the manufacturer's protocol (Gibco/BRL). The quality of the synthesized cDNA was controlled by RT-PCR amplification of GAPDH using primers KN-45 (CCA CCC ATG GCA AAT TCC ATG GCA) and KN-46 (TCT AGA CGG CAG GTC AGG TCC ACC). CD21 expression was tested by RT-PCR amplification of exon 9-12b using primers KN-127 (ACC ATC CGT TGT ACA AGC AAT G) and KN-128 (CAG TCA ACT GGT ACC CAT CTT G).

Flow cytometric analysis

Cells (1–5×10^5) were transferred to a 96-well microtiter plate and centrifuged for 5 min at 280 *g*. The supernatant was disposed and the cell pellet resuspended in FACS buffer (1×PBS/2% FCS/0.01% sodium azide) containing fluorochrome-labeled antibodies. Cells were stained in the dark on ice for 20 min. Cells were then washed with FACS buffer and resuspended in 200 μl FACS buffer containing 0.5 μg/ml propidium iodide. Cells were analyzed using a FACScan (Becton Dickinson, Heidelberg, Germany) and a microtiter plate reader developed in our lab (52). Antibodies used in flow cytometric analysis were CD19 (Dako, Hamburg; clone HD37, R-phycoerythrin-conjugated) and CD21 (Coulter-Immunotech; clone BL-13, FITC-conjugated).

Results

The CD21 CpG island is methylated in pro-, pre- and intermediate B lymphocytes

First, we analyzed the methylation status of the CD21 CpG island in pro- and pre-B lymphocytes by Southern blot analysis. As others before, we employed the methylation-sensitive restriction enzymes *Hpa*II and *Eco*72I in our analysis. The probe used to visualize the CD21 CpG island region (Fig. 1A) detected a 3.4 kb *Eco*RI band in all cell lines included in our analysis. We analyzed genomic DNA from the pro-B cell lines RS4;11, Nalm16, KM3 and REH, and the pre-B cell lines OB5, 697 and Nalm6 (Fig. 1B). As can be seen in Fig. 1(B), the DNA of all pro- and pre-B cell lines was partially methylated at the *Hpa*II–*Msp*I sites of the CD21 promoter region. Two *Eco*72I sites within the 3.4 kb *Eco*RI fragments were monitored for methylation too. Using *Eco*RI–*Eco*72I double digests, a 480 band is indicative for the absence of methylation, while methylated DNA would give rise to the 3.4 kb *Eco*RI band

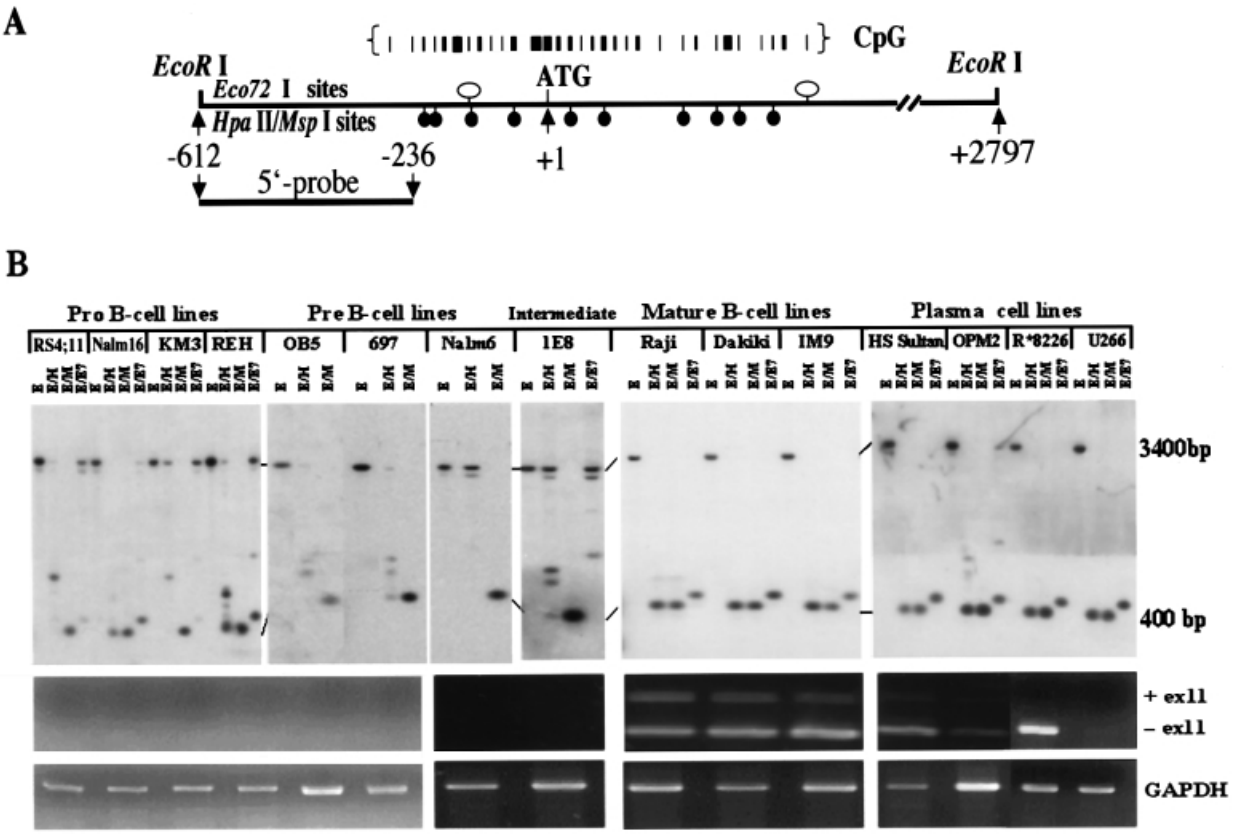

Fig. 1. A map of the 3.4-kb *Eco*RI fragment is shown (A) indicating the *Eco*72I (open circles) and the *Hpa*II–*Msp*I sites (closed circles). CpG dinucleotides within the borders of the CpG island are represented by vertical bars. The position of the ATG start codon of CD21 is marked by an arrow. The probe used for the Southern blots is marked with a bar (5′ probe). Southern blot analysis of B lymphoid cell lines representing the various developmental stages of B cell maturation. Genomic DNA of the cell lines was subjected to restriction digest with *Eco*RI (E), *Eco*72I (E7), *Hpa*II (H) and *Msp*I (M) as indicated. Southern blots were probed with the p21pro insert as indicated in (A). The positions of the restriction sites are with respect to the position of the ATG: *Hpa*II–*Msp*I: –219; –187; –128; –54; +45; +97; +246; +306; +346; +393. *Eco*72I: –136; +455. Pro- and pre-B lymphocytes contain a partially methylated CpG island while mature and plasma B lymphocytes do not. To investigate CD21 mRNA expression of these cells we have performed RT-PCR using primers displaying the region surrounding the alternatively spliced exon 11. PCR products are shown below the Southern blots with GAPDH controls underneath.

only. All pro- and pre-B cell lines contain partially methylated *Eco*72I sites (Fig. 1B). Expression of CD21 in these cell lines was analyzed by RT-PCR and flow cytometry. As expected, all of the pro- and pre- B cells do not express CD21, while mature B lymphocytes do. The 1E8 cell line represents an intermediate step in B lymphocyte maturation. The cell line expresses both conventional and surrogate light chains. Conventional light chains form, together with Ig heavy chains, the BCR on the cell surface, whereas the surrogate light chains are confined to the cytoplasm (53). This cell line does not express CD21 as determined by RT-PCR (54) (Fig. 1B). Southern blot analysis for methylation of the *Hpa*II–*Msp*I and *Eco*72I recognition sites within the 3.4 kb *Eco*RI fragment revealed that the sites are partially methylated.

Mature and plasma B lymphocytes are not methylated within the CD21 CpG island

When pre-B cells differentiate into mature B cells they do express Ig heavy chains in conjunction with Ig light chains and CD21 on the cell surface. We have used the mature B cell lines Raji, Dakiki and IM9 to perform methylation analysis of genomic DNA. We found that in these cells all *Hpa*II and *Eco*72I sites within the 3.4-kB *Eco*RI fragment were not methylated as visualized by the appearance of the 400-bp band in both the *Hpa*II–*Eco*RI and *Msp*I–*Eco*RI as well as the 480-bp band in the *Eco*72I–*Eco*RI restriction digest.

RT-PCR and flow cytometry analysis revealed that the mature B lymphoid cell lines expressed CD21 mRNA and surface protein respectively (Fig. 1B).

To complete the picture of human B cell development we have analyzed the plasma cell lines HS Sultan, OPM2, RPMI8226 and U266. U266 does not express CD21 while unexpectedly we found expression CD21 mRNA by RT-PCR in the other cell lines. However, immunofluorescence analysis of the cell lines using a monoclonal anti-CD21 antibody did not reveal detectable CD21 on the cell surface except for HS Sultan cells (data not shown). When we performed methylation analysis we found no methylation within the entire 3.4 kb *Eco*RI fragment surrounding the CD21 promoter using *Eco*RI–*Hpa*II, *Eco*RI–*Msp*I and *Eco*RI–*Eco*72I double digests (Fig. 1B).

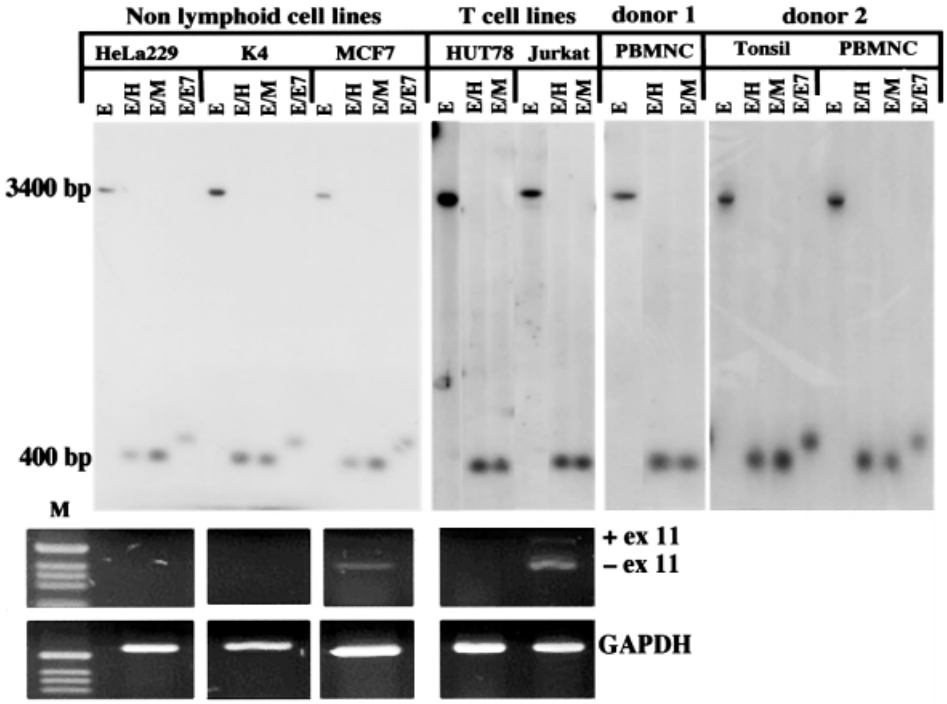

Fig. 2. Analysis of non-lymphoid primary mononuclear cells and T lymphoid cells by Southern blot analysis revealed that the CD21 CpG island is not methylated at the *Eco*72I (E7) and *Hpa*II (H)–*Msp*I (M) sites. Double digests were done with *Eco*RI (E). RT-PCR analysis for CD21 expression using primers displaying the region surrounding the alternatively spliced exon 11 and GAPDH control is shown below. Unexpectedly, the mammary carcinoma cell line MCF-7 expressed CD21 mRNA while other non-lymphoid cells did not. Both activated (tonsil) and resting lymphocytes (PBMC) are demethylated.

Methylation status of the CD21 CpG island in primary mature and activated mononuclear cells

Peripheral blood B lymphocytes are mature stage lymphocytes and do express CD21 as do T cells from the peripheral blood (55). We have used Ficoll gradient purified peripheral blood mononuclear cells (PBMC) from a healthy donor (donor 1) and analyzed the methylation status of the CD21 CpG island. As depicted in Fig. 2, the PBMC containing both B and T lymphocytes are demethylated as determined by restriction analysis as in Fig. 1. We also analyzed cells from a second donor (donor 2) where mononuclear cells from excised tonsils and from blood of this person could be investigated. All samples were found free of methylated sites in the area examined (Fig. 2). Thus the CD21 CpG island of both resting and activated primary B and T lymphocytes is not methylated.

The CD21 gene CpG island in T lymphocytes and non-lymphoid cells was not methylated

We and others have shown that T lymphocytes do express CD21 mRNA to levels almost comparable to B lymphocytes (54,55). We analyzed the CD21-expressing Jurkat T lymphoid cell line and HUT78, which do not express CD21, for methylation of the CD21 CpG island. As depicted in Fig. 2, both are free of methylation at the *Hpa*II and *Eco*72I sites. Furthermore, the majority of the lymphocytes among the purified PBMC were T lymphocytes. Since no methylation could be observed in the analysis shown in Fig. 2, we conclude that primary T lymphocytes from the blood contain non-methylated CD21 CpG islands too.

When B lymphocytes are methylated in early stages and only demethylate when they mature, what do we expect from non-lymphoid cells? We have analyzed the methylation pattern of the same *Hpa*II and *Eco*72I sites of the 3.4-kb *Eco*RI promoter fragment in the cell lines HeLa229 (cervix carcinoma), K4 (SV40-transformed synovial fibroblast) and MCF-7 (mammary carcinoma), and found (Fig. 2) that the CD21 CpG island is free of methylation at the sites analyzed.

Inhibition of methylation and histone deacetylation leads to expression of the CD21 gene in pro-B cells

DNMT activity can be blocked irreversibly using the base analogue 5-aza-C. We applied this drug to cell cultures of Nalm16 and KM3 cells for 72 h, and prepared total RNA. Inhibition of DNMT resulting in CD21 expression should lead to loss of methylated CpGs in the CD21 CpG island. To analyze this we have treated KM3 cells with 5-aza-C and performed Southern blot analysis. As can be seen in Fig. 3 treatment of the cells resulted in loss of methylation indicated by the appearance of the 400-bp *Hpa*II, the 480-bp *Eco*72I band and the loss of the intermediate size bands reflecting partial methylation. RT-PCR revealed that treatment of the cells resulted in CD21 expression, whereas no CD21 expression could be detected in the control cells without the drug (Fig. 4A and B). Furthermore, the transcriptional activation also led to the simultaneous expression of both long and

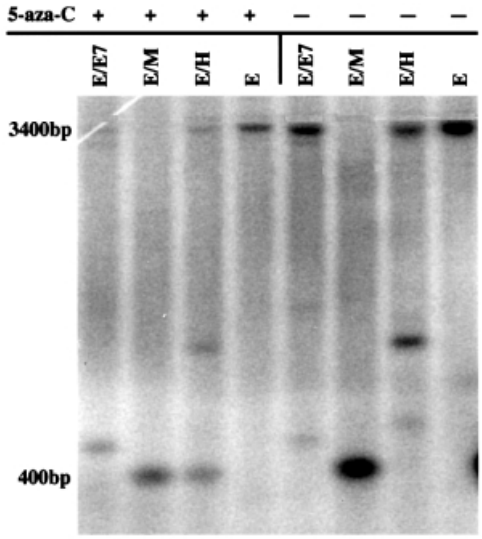

Fig. 3. We performed Southern blot analysis to look for the methylation status in KM3 cells with and without 5-aza-C treatment. As indicated by the appearance of the 400 bp *Eco*RI–*Hpa*II and the 480 bp *Eco*RI–*Eco*72I bands in the treated cells, demethylation occurs in the CD21 CpG island.

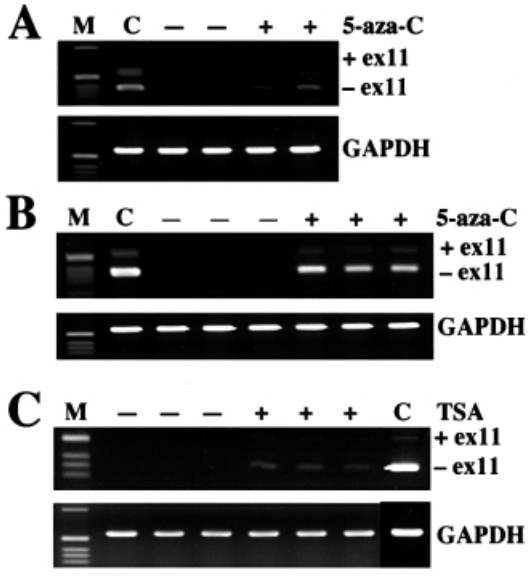

Fig. 4. In order to analyze whether methylation is a cause of CD21 silencing in early B lymphocytes we have applied 5-aza-C and TSA to Nalm16 (A and C) and KM3 (B) cells as indicated. Cells were treated with the drugs and total mRNA prepared. RT-PCR was performed using primers amplifying a region surrounding exon 11. Nalm16 cells expressed CD21 upon treatment with 5-aza-C and TSA (A and C); KM3 cells expressed CD21 upon treatment with 5-aza-C. Thus both mechanisms orchestrate to control CD21 expression. A GAPDH control is shown underneath the CD21 PCR products for each PCR. Experiments shown with Nalm16 were performed in duplicate for the 5-aza-C treatment and in triplicate for all other experiments. In particular, both forms of CD21 mRNA were found to be co-expressed.

short isoforms of CD21 generated by alternative splicing, as can be found in all cell lines expressing CD21 (Fig. 4A–C) (54,55).

Histone deacetylation occurs at lysine residues on the N-terminal tails of the histones, thereby increasing their affinity for DNA. As a consequence, histone deacetylation alters nucleosomal conformation, which decreases the accessibility of transcriptional regulatory proteins to chromatin templates. It is known that DNA methylation and histone deacetylation are functionally coupled as the major methyl-cytosine-binding-protein MeCP2 recruits a histone deacetylase when bound to methylated DNA (26). Therefore, since DNA demethylation resulted in expression of the CD21 gene, we could expect to obtain a similar response blocking histone deacetylase function. In order to analyze whether CD21 expression is controlled by this mechanism we have used TSA to inhibit histone deacetylation. The drug was applied at 300 ng/ml to cell cultures of Nalm16 cells for 72 h. Control cells were kept in parallel cultures with the solvent DMSO. Indeed treatment of the cells resulted in activation of CD21 expression (Fig. 4C). Thus CD21 expression is controlled likely by both activities of methyltransferase and histone deacetylase.

Discussion

CD21 participates in the activation and survival of B lymphocytes (17). During ontogeny of B lymphocytes the expression of the CD21 gene is confined to the stage of mature B lymphocytes (20,21). The presence of a CpG island in the CD21 promoter tempted us to investigate its role in CD21 expression control. Methylation of CpG dinucleotides is a widely used mechanism to control gene expression (27). In vertebrates, 60–90% of all CpGs are methylated, while CpGs in functional and active promoters are mostly demethylated (26). The CD21 CpG island contains 10% CpG dinucleotides (in an area of 674 bp surrounding the ATG start codon) unlike the 0.3–0.6% found in the human genome (26).We show here that immature pro- and pre-B cells contain a methylated CD21 CpG island, whereas mature B lymphocytes display a CpG island containing a 3.4-kb *Eco*RI fragment free of methylation, at least at the restriction enzyme recognition sites analyzed. We investigated whether methylation of the CD21 gene promoter is functionally significant and used the DNMT inhibitor 5-aza-C. This drug caused a reduction in CD21 promoter methylation and induced the expression of the CD21 gene. It is known that methylated CpG islands are binding sites for methylated cytosine-binding proteins such as MeCP2 which interacts with protein complexes containing histone deacetylase activity. Therefore we analyzed whether histone deacetylation might be part of the expression control of CD21. For this purpose we used TSA to inhibit histone deacetylases. We found that treatment led to the induction of CD21 transcription, probably by converting adjacent chromatin to a more open configuration. Although suggestive, our findings do not prove a causal link between DNA methylation, histone acetylation and gene expression.

Other possibilities are to be considered. There are proteins other than MeCPs known which can actively influence demethylation such as NF-κB (30) and prevent *de novo* methylation such as Sp1 (56). We found Sp1 and NF-κB

binding sites in the silencer and promoter region of CD21. Work to delineate the genetic element involved in control of the CD21 CpG island methylation are underway.

An interesting finding is that the expression of CD21 in early lymphocytes induced by TSA and 5-aza-C lead to both alternatively spliced forms of CD21. This suggests that the splicing factors are already present even in the absence of a particular differentiation program for CD21 expression. Either the factors involved are activated by both TSA and 5-aza-C treatment or they already exist in the absence of CD21 expression.

Acknowledgements

We would like to thank Rolf Knippers, Andreas Radbruch and Samuel Solomon for critical comments on the manuscript, Brigitte Schanze for help in manuscript preparation, Dr Greil for sending us the cell lines REH, KM3, HS Sultan, RPMI8226, U266 and OPM2, Dr Elisa May for HeLa229 and MCF7, and Dr Gordana Bothe from GATC, Konstanz, for help with the sequence compilation. This work was supported in part by grant Il32/2-1 through the Deutsche Forschungsgemeinschaft and AFF grant 4/99 through the University of Konstanz to H. I.

Abbreviations

5-aza-C	5-aza-2′-deoxycytidine
DNMT	DNA methyltransferase
MeCP	methylcytosine-binding protein
PBMC	peripheral blood mononuclear cell
TSA	Trichostatin A

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