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# Genomic structure, functional comparison, and tissue distribution of mouse *Cd59a* and *Cd59b*

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**Abstract.** CD59 is a crucial complement regulatory protein that inhibits the terminal step of the complement activation cascade by interfering with the binding of C9 to C5b-8, thus preventing the formation of the membrane attack complex (MAC). We recently reported that the mouse genome contains two *Cd59* genes, while the human and rat genomes each contain only one *Cd59* gene (Qian et al. 2000). Here, we describe the genomic structure, comparative activity, and tissue distribution of these two mouse genes, designated *Cd59a* and *Cd59b*. The mouse *Cd59* genes encompass a total of 45.6 kb with each gene having four exons. *Cd59a* spans 19 kb, and *Cd59b* spans 15 kb, with approximately 11.6 kb of genomic DNA separating the two genes. The overall sequence similarity between *Cd59a* and *Cd59b* is approximately 60%. The sequence similarity between exon 2, exon 3, and exon 4 and the respective flanking regions between the two genes is over 85%, but exon 1 and its flanking regions are totally different. Comparative studies of the activity of both genes as inhibitors of MAC formation revealed that *Cd59b* has a specific activity that is six times higher than that of *Cd59a*. Using polyclonal antibodies specific to either Cd59a or Cd59b, we showed that Cd59a and Cd59b are both widely expressed in the kidneys, brain, lungs, spleen, and testis, as well as in the blood vessels of most mouse tissues. Interestingly, testicular Cd59a appeared to be expressed exclusively in spermatids, whereas Cd59b was expressed in more mature sperm cells. These results suggest that even though Cd59a and Cd59b are expressed in multiple tissues, they may play some different roles, particularly in reproduction.

## Introduction

Formation of the membrane attack complex (MAC) is the final step of the complement activation cascade. The MAC is a heteropolymeric complex with the capacity to insert into cell membranes and form a transmembrane pore. Diffusion of ions and water through the MAC-pore can lead to colloidosmotic swelling and lysis of the target cell. For this reason, the complement system has been historically depicted as an effector of the immune system and a defense barrier against bacteria and/or foreign cells. However, we have shown that the same MAC that can induce lethal colloidosmotic swelling in Gram-negative bacteria and erythrocytes can generate significant but reversible changes in the membrane permeability of autologous cells that mediate physio/pathological signals without compromising the cells' viability (Halperin et al. 1993; Nicholson-Weller and Halperin 1993). For example, we have documented that insertion of the MAC into endothelial cells results in the release of growth factors that auto- and paracrinically stimulate cell proliferation. MAC insertion into target cell membranes also activates other pathways of intracellular signaling including $Ca^{++}$-sensitive and $Ca^{++}$-insensitive protein kinase C (PKC), and the adenyl cyclase system (Carney et al. 1985, 1990; Cybulsky et al. 1990). In addition, insertion of the MAC triggers the release of procoagulant activity from platelets, stimulates oxygen radical generation by leukocytes, and releases IL-1 from glomerular mesangial cells (Sims et al. 1988; Hallett et al. 1981; Lovett et al. 1987). Consistent with the findings that support a role of the MAC as a mediator of pathophysiological signals, MAC deposition has been documented in a variety of immune and nonimmune proliferative diseases including atherosclerosis, arthritis, and glomerulonephritis as well as in neurodegenerative conditions such as Alzheimer's disease (Yang et al. 2000). Recently, we have uncovered a link between glycation-inactivation of CD59, increased MAC deposition, and the vascular proliferative complications characteristic of human diabetes (Acosta et al. 2000). Glycation inhibits the function of human CD59 because it expresses a glycation motif formed by residues K41 and H44 (Acosta et al. 2000). Consistently, an analysis of nerve biopsies from patients with diabetic neuropathy has shown that deposition of MAC and other activated complement proteins in endoneurial microvessels is the predominant marker that differentiates diabetic neuropathy from other forms of neuropathy (Rosoklija et al. 2000).

The growing evidence for a prominent role of complement and the MAC in the pathogenesis of human diseases explains the evolution of a series of proteins that tightly restrict the formation of autologous MAC. In addition to an array of fluid phase complement inhibitors, three membrane proteins, DAF, MCP, and CD59, interrupt specific steps of the activation cascade and prevent the pleomorphic pathological responses that may result from excessive autologous MAC formation. Several observations indicate that in most species CD59 plays a prominent role in restricting complement-induced phenomena. Indeed, an individual who reportedly has an isolated germline CD59 deficiency exhibits a paroxysmal nocturnal hemoglobinuria (PNH) phenotype (complement-mediated hemolytic anemia) that is not seen in individuals with isolated red blood cell decay accelerating factor (DAF) deficiency nor in DAF

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knockout mice (Yamashina et al. 1990; Lin et al. 1988; Telen and Green 1989; Reid et al. 1991; Sun et al. 1999).

Human CD59 is an 18- to 20-kDa glycoprotein that is present in all circulating cells, as well as in the epithelia, endothelia, and almost all tissues. Humans have only one copy of the *CD59* gene, which contains four exons and spans 26 kb (Petranka et al. 1992; Qian et al. 2000). Rats, sheep, and pigs also each have a single *CD59* gene that has been cloned and functionally characterized (Matsuo et al. 1994; van den Berg et al. 1993, 1995). In contrast, mice express two *Cd59* genes, known as *Cd59a* and *Cd59b. Cd59a* was described in 1997, and *Cd59b* was recently cloned and functionally characterized (Qian et al. 2000; Powell et al. 1997). Both *Cd59a* and *Cd59b* are GPI-anchored cell membrane proteins and share 83% nucleotide similarity and 60% protein identity. mRNA encoding for *Cd59b* is highly expressed in the testis, while *Cd59a* mRNA is highly expressed in the heart, kidneys, liver, and lungs, but only minimally in the testis (Qian et al. 2000). We report here the genomic structure of the *Cd59a* and *Cd59b* genes and compare their functional characteristics and tissue distribution.

## Materials and methods

*Materials.* General cell culture reagents, lipofectamine, and the trizol reagent were from Gibco (Grand Island, N.Y.); Chinese hamster ovary cells (CHO) from ATCC (Bethesda, Md.); 129/SV murine lambda FIX™ genomic library from Stratagene (La Jolla, Calif.); mouse multiple tissue cDNA panel and NucleoTrap@mRNA kit from Clontech (Palo Alto, Calif.); plasmid, lambda, and bacterial artificial chromosomes (BAC) DNA purification kits from Qiagen (Valencia, Calif.); PCR and pAlter vectors from Promega (Madison, Wis.); pcDNA 3 from Invitrogen (Carlsbad, Calif.); "Down to the well"™ mouse BAC pools from Genome Systems (St. Louis, Mo.; BCECF-AM from Molecular Probes (Eugene, Ore.); Northern nylon membrane from NEN™Life Science Products (Boston, Mass.); IgG fraction of rabbit anti-hamster lymphocyte from Inter-cell Technologies (Hopewell, N.J.); FITC-labeled mouse Chr 2-painting probe from Cambio (Cambridge, England); Cy3-avidin from Amersham (Piscataway, N.J.); 3.3′-diaminobenzidine (DAB)-methyl green from Sigma Chemical Company (St. Louis, Mo.); SulfoLink Coupling Gel Support from Pierce (Rockford, Ill.); and universal power blocking reagent from BioGenexm (San Ramon, Calif.).

*Phage library screening.* $^{32}$P randomly labeled full-length cDNA of *Cd59a* and *Cd59b* was used as a probe to screen a 129/SV murine lambda FIX™ genomic library according to the manufacturer's instructions. Positive clones were identified through secondary and tertiary screening, and phage DNAs were prepared by the liquid culture method by using the Qiagen Lambda Midi Kit.

*DNA sequencing.* Walking primers were designed with primer design software. Primers were synthesized and used for further sequencing at the Tufts Core Sequencing Facility (Boston, Mass.). Sequences were analyzed and aligned by using a 3.1 sequencer (Gene Codes Corporation, Ann Arbor, Mich.). The genomic sequence downstream from exon 4 of *Cd59a* was obtained by inverse PCR (Yoshitomo-Nakagawa et al. 1997). Inverse primers (forward: AGGGTAGAACACCTATAGGTT; reverse: ATCCGTCACTTTTGTTACAC) and Taq DNA-polymerase were used to amplify templates from the self-ligation products of *Hin*dIII-, *Bam*HI-, and *Eco*RI-digested mouse genome fragments. Specific PCR products were cloned into the Promega PCR vector and sequenced.

*Isolation of BAC genomic clone.* "Down to the well"™ mouse BAC pools were screened with *Cd59b* specific genomic primers (forward: GATCCCAATCAATGTATGCTT; reverse: TCAGTATGTCCCTAGTCA). One positive BAC was selected and confirmed as a genomic clone containing *Cd59b* by BAC direct sequencing with *Cd59b* specific genomic primers.

*Fluorescence in situ hybridization (FISH).* Metaphase chromosomes were prepared from normal male mouse embryonic fibroblasts by using established procedures (Nesbitt and Francke 1973). Chromosomes were denatured in 70% formamide, 2× SSC at 70°C for 2.5 min, dehydrated sequentially in 70%, 90%, and 100% ethanol, and dried by air. DNA from a mouse BAC clone carrying the *Cd59b* gene was nick-translated with biotin-16-dUTP (BoehringerMannheim, Germany), denatured at 70°C for 8 min, and applied to the slides. The slides were incubated at 37°C for 16 h, followed by two 5-min washes in 50% formamide, 2× SSC at 43°C, and two additional 5-min washes in 0.5× SSC at 43°C. The biotinylated probe was detected with Cy3-avidin. Slides were subsequently hybridized with an FITC-labeled mouse chromosome-specific painting probe for positive identification of mouse Chr 2.

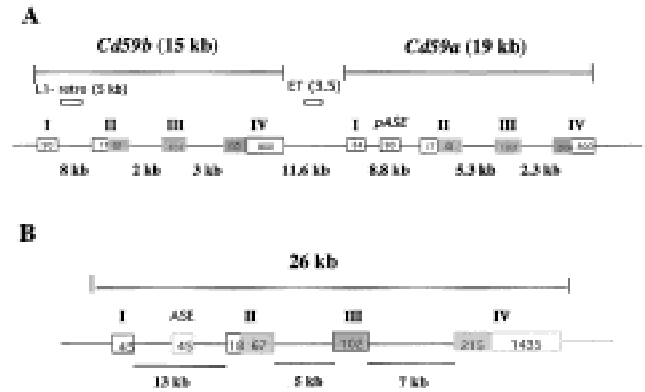


**Fig. 1.** Comparison of the structural organization of mouse *Cd59* and human CD59. Translated exons are shown as shaded rectangles and the 5′ and 3′ untranslated regions are illustrated by white rectangles. The figure also shows the number of nucleotide base pairs in each exon. **A.** Structural organization of mouse *Cd59:* A 5-kb L1-retrotransposon repeat sequence (L1-retro) between exon 1 and 2 of *Cd59b* and a 3.5-kb mouse early transposon (ET) sequence between *Cd59a* and *Cd59b* are shown (superscripted rectangles). A putative alternative splice exon (*pASE*) of *Cd59a* identical to that of *Cd59b* exon 1 lies between exon 1 and 2 of *Cd59a*. **B.** Structural organization of human *CD59:* Alternative splice exon (ASE) lies between exon 1 and 2 of human CD59.

*Construction of FLAG-tagged Cd59a and Cd59b.* FLAG-tagged *Cd59a* and *Cd59b* constructs have been described (Qian et al. 2000). Briefly, the 24 nucleotide FLAG-encoding sequence (GACTACAAGGACGACGATGACAAG) was inserted into the coding region of *Cd59a* and *Cd59b* after the signal peptide and before the second amino acid in the mature Cd59 protein [threonine (*Cd59a*) or lysine (*Cd59b*)]. After cleavage of the signal peptide in the endoplasmic reticulum (ER), these constructs yield N-terminal-tagged Cd59a and Cd59b proteins that function as MAC inhibitors exactly like the wild-type proteins (Qian et al. 2000).

*Cell culture and transfection.* CHO cells were grown in F12 medium supplemented with 10% heat-inactivated fetal bovine serum. DNA transfection was carried out with lipofectamine following the manufacturer's instructions. To confer G418 resistance, cells were co-transfected with pAlter or pAlter-FLAG-*Cd59a* or *Cd59b* and pCDNA3 (5 μg pAlter or pAlter-FLAG-*Cd59* plasmid and 100 ng pCDNA3). Two days after transfection, 800 μg/ml G418 was added to select for transfected cells. Individual colonies were transferred into a 12-well plate and expanded after approximately 20 days. A drug-resistant clone from pAlter-transfected cells was used as a control in later assays.

*FACS analysis.* Transfected CHO cells grown to near-confluence were detached with cell dissociation solution and re-suspended to a density of $\approx 2 \times 10^5$ cells/tube. Cells were incubated with the primary antibody for 30 min at room temperature, washed three times with PBS/BSA (3%) buffer, and incubated for 30 min with an FITC-conjugated secondary antibody. They were washed in PBS three times before analysis of fluorescence intensity by using a Becton Dickinson FACScan.

*Lysis protection assay.* Activity of mouse *Cd59* proteins expressed in CHO cells was evaluated by a modification of the dye release assay de-

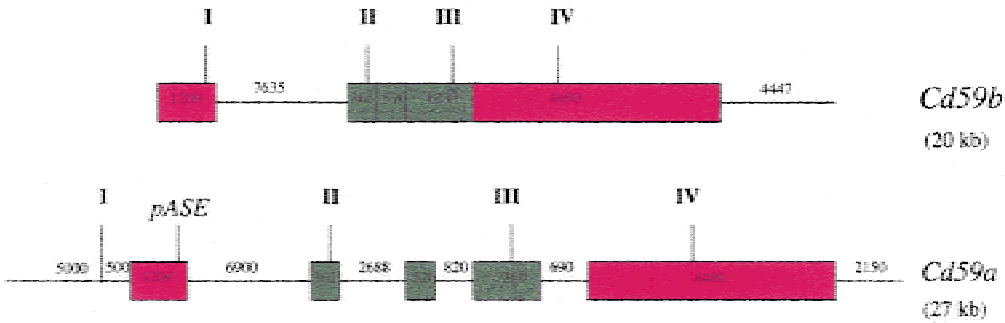


**Fig. 2.** Comparison of genomic sequence of mouse *Cd59.* The overall genomic sequence similarity between *Cd59a* and *Cd59b* is 60%. The regions of very high sequence similarity, which are mainly clustered around the exons, are highlighted (red squares, 85–90%; green squares, 90–96%). The figure also shows the number of nucleotide base pairs in each homologous regions.

scribed by Qian et al. (2000). Briefly, approximately $2 \times 10^4$ cells/well were seeded in 96-well plates. After reaching 90% confluence, they were loaded with the fluorescent dye BCECF-AM (20 μM) at 37°C for 30 min. After washing several times to remove unincorporated dye, cells were sensitized with 50 μl of an IgG fraction of rabbit anti-hamster lymphocyte (4 mg/ml in HBSS/1%BSA). The antibody-sensitized cells were then exposed for 30 min at 37°C to a source of complement diluted in HBSS/1%BSA. Supernatants were collected to determine the fluorescent dye content as a measure of cell lysis. The percentage cell lysis was calculated from the ratio of the fluorescent dye released into the supernatant over the fluorescent dye released after total cell lysis.

*Multiple tissue RT-PCR.* Specific PCR primer pairs for *Cd59a* and *Cd59b* were made based on the *Cd59* cDNA sequence (Qian et al. 2000). Their specificity was confirmed by amplification of the *Cd59a* and *Cd59b* plasmids. The specific primers for *Cd59a* were: forward primer, 5′ TGGTTTCTTCATGCAATATGAACAG 3′, and reverse primer, 5′ TAGGCAGTGAAAACAATGTG 3′. The specific primers for *Cd59b* were: forward primer, 5′ ACAGTGTTGGAAACTTTCGG 3′, and reverse primer, 5′ TACTCCTTCAAAGACGCAAC 3′. To survey expression in multiple mouse tissues, PCR was performed with a mouse multiple tissue cDNA panel as the template and each *mCd59* specific primer pair. PCR products were analyzed on 1% agarose gel.

*Northern blot.* Total RNAs from the brain, heart, and kidneys of three different mouse strains (129/sv, CAST/Ei, and C57BL/6J) were isolated using the trizol reagent. Tissue mRNAs were then purified by using the NucleoTrap@mRNA kits. Messenger RNA samples (10 μg each lane) were separated on a 1.0% formaldehyde-agarose gel and transferred onto nylon membrane via capillary action overnight in 20× SSC. The membrane was cross-linked under UV and hybridized with a $^{32}$P-labeled *Cd59a* or *Cd59b* specific probe. After hybridizing with the *Cd59b* probe, the membrane was stripped by boiling in 0.1× SSC/0.1% SDS and rehybridized with the *Cd59a* probe. Both cDNA probes were random labeled with $^{32}$P-dCTP. Northern hybridizations were carried out in Church buffer (0.25 *M* $Na_2HPO_4$, 5% SDS, 1% BSA) at 60°C, overnight. The membrane was washed, first in 2× SSC/0.1% SDS at 55°C for 15 min, then in 0.3× SSC/0.1% SDS at 60°C for 1 h, and exposed to X-ray film.

*Generation of antibodies.* The QRCWKQSDAHGEIIMDQLEETKLKF peptide sequence from Cd59a and the QQCWKLSDANSNYIMSRLDVAGIQS peptide sequence from Cd59b were synthesized, conjugated to keyhole limpet hemocyanin (KLH), and injected into two white New Zealand female rabbits for antibody production by Biosynthesis Inc. (Lewisville, Tex.) (Harlow and Lane 1988). Anti-Cd59a and anti-Cd59b antibodies were affinity purified by immobilizing the antigen peptides on a SulfoLink Coupling Gel and eluting with 0.1 M glycine, pH = 3.

*Immunohistochemistry.* Four-micron-thick frozen tissue sections were cut, mounted onto Superfrost plus slides, and fixed in cold acetone for 10 min in the cryostat. The sections were washed twice for 3 min each with PBS and blocked with universal power blocking reagent for 15 min in a humid chamber. The sections were then incubated with rabbit polyclonal antibodies against Cd59a (1:200 dilution) and Cd59b (1:100 dilution) for 1 h. The slides were washed with PBS, and the secondary antibody (Vectastain Elite ABC Kit, Vector Laboratories, Inc., Burlington, Calif.) was applied according to the company instructions. Slides were rinsed and the sections were developed by using 3.3′-diaminobenzidine (DAB) as substrate and counterstained with methyl green.

## Results

*Genomic structure of Cd59a and Cd59b genes.* To define the genomic structure of *Cd59a* and *Cd59b*, $1.5 \times 10^6$ independent colonies of a mouse genomic phage library were screened (approximately nine mouse genome equivalents). Ten positive plaques were identified and confirmed by two additional rounds of screening. Three overlapping phages were completely sequenced by using the primer walking strategy, and the other seven phage-end fragments were partially sequenced with T3 and T7 primers. Analysis of the phage sequences obtained strongly indicated that the selected 10 phage clones covered a continuous sequence. Because the 43.6-kb sequence obtained from these 10 phage clones ends at the third intron of *Cd59a* and does not include exon 4, we used inverse PCR to sequence an additional 3.4 kb downstream of the third intron of *Cd59a.* This combined sequencing strategy provided a 47-kb sequence in the mouse genome which contained the whole genomic sequence of *Cd59a* and *Cd59b* (GenBank accession number AF292401). The mouse *Cd59* genes span a total of 45.6 kb; *Cd59a* and *Cd59b* respectively span 19 kb and 15 kb and are separated by approximately 11.6 kb of genomic DNA. Each of the two mouse *Cd59* genes has four exons and independent flanking regions (Fig. 1A). Together, these data indicate that *Cd59a* and *Cd59b* are two independent genes and not the result of the alternative splicing of a single gene.

The overall sequence similarity between the *Cd59a* and *Cd59b* genes is approximately 60%. There are regions of high (over 85%) similarity which are mainly clustered at and around exons 2, 3, and 4. In contrast, the exon 1 sequences in *Cd59a* and *Cd59b* are highly divergent (Fig. 2; Qian et al. 2000). A putative alternative splicing exon (*pASE*) identical to that of *Cd59b* exon 1 is found in the genomic region between exons 1 and 2 of *Cd59a*, 1.6 kb downstream from *Cd59a* exon 1 (Fig. 1A). Analysis of the genomic sequence reveals that there is a 5.0-kb L1-retrotransposon repeat sequence between exons 1 and 2 of *Cd59b*, and that there is a 3.5-kb mouse early transposon sequence between *Cd59a* and *Cd59b* (Fig. 1A).

*Cd59a* is located on mouse Chr 2 (Powell et al. 1997). Linkage analysis of *Cd59b* and *Cd59a* in the mouse genome indicated that *Cd59b* is also located on Chr 2. FISH analysis with a *Cd59b* specific probe localized *Cd59b* to a single site on a pair of Chrs 2 (Fig. 3A, 3B). The close proximity of *Cd59a* and *Cd59b* was further confirmed by partially sequencing the BAC spanning *Cd59b* (data not shown).

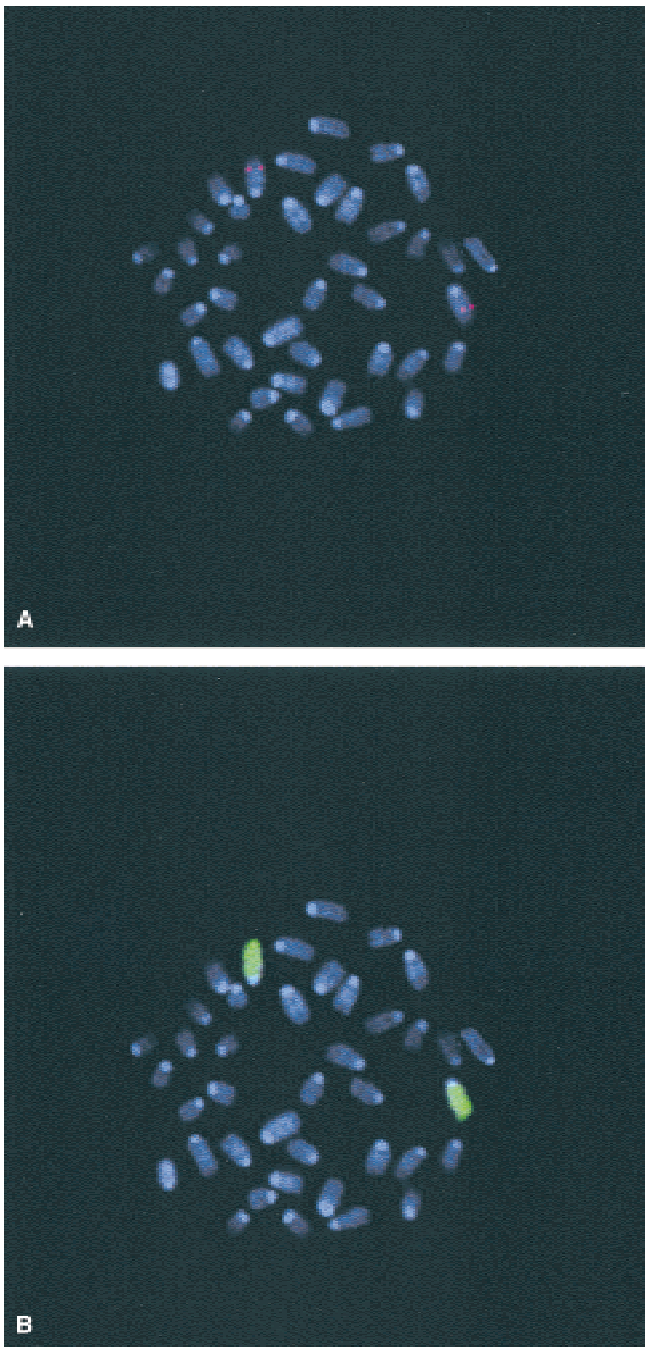


**Fig. 3.** Fluorescence in situ hybridization (FISH) analysis. **A.** A BAC clone containing the *Cd59b* gene was hybridized to the chromosomes from a male mouse. Cy3 detection of the probe produces red signals on a pair of homologous chromosomes. **B.** These chromosomes were subsequently identified as mouse Chr 2 with an FITC-labeled mouse Chr 2 paint probe (green).

*Functional comparison of Cd59a and Cd59b.* To compare the functional activity of Cd59a and Cd59b, both proteins harboring a FLAG sequence at the N-terminus (FLAG-Cd59) were expressed at different densities in CHO cells by using the pAlter vector. Membrane expression of both Cd59 proteins in transfected CHO cells was confirmed with anti-FLAG antibodies and fluorescent secondary antibodies (data not shown). Because mouse Cd59 exhibits cross-protection against human complement (Powell et al. 1997), human serum was used as a source of complement to compare the activity of Cd59a and Cd59b. Antibody-sensitized CHO

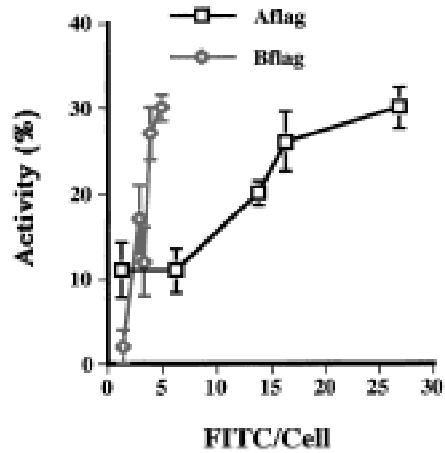


**Fig. 4.** Functional comparison of mouse *Cd59a* and *Cd59b*. The percentage release of BCECF fluorescent dye from pre-loaded CHO cells measures complement mediated lysis. The reduced lysis of *Cd59* expressing cells is the consequence of *Cd59* activity. The density of *Cd59* molecules expressed on the membrane of the CHO cells was determined by FACS analysis using anti-FLAG specific antibodies. The specific activity of each *Cd59* was calculated from the slope of the curve obtained by plotting the protective capacity of each *Cd59* vs. the number of *Cd59* molecules per cell.

cells expressing either Cd59a or Cd59b were loaded with the BCECF fluorescent dye before exposure to 10% human serum. The amount of dye released by complement-mediated lysis from CHO cells transfected with vector alone minus the dye released from the Cd59 expressing cells provides a measurement of the activity of Cd59 on the cell surface. The results show that both Cd59a and Cd59b were active in protecting antibody-sensitized CHO cells from lysis by human complement. To estimate the specific activity of each Cd59 protein, the density of Cd59 molecules expressed on the membrane of the CHO cells was determined by FACS analysis using anti-FLAG specific antibodies. The specific activity of each Cd59 was calculated from the slope of the curve obtained by plotting the protective capacity in the dye release assay vs. the number of Cd59 molecules per cell (Fig. 4). Analysis of the relative activities of each protein showed that Cd59b is approximately six times more active than Cd59a, at least against human complement. A similar comparison with mouse complement was not performed because mouse serum is cumbersome to manipulate as a source of complement.

*Tissue distribution of* Cd59a *and* Cd59b *mRNAs.* A mouse multiple tissue cDNA panel was screened by PCR by using specific *Cd59a* and *Cd59b* primer pairs. The specificity of the primer pairs was demonstrated by amplifying relevant regions of the *Cd59* genes with *Cd59a* and *Cd59b* plasmids as templates. The *Cd59a*-specific primer pair amplified the *Cd59a* template but not the *Cd59b* template, while the *Cd59b*-specific primer pair amplified the *Cd59b* template but not the *Cd59a* template. The PCR results indicated that both *Cd59a* and *Cd59b* mRNAs are present in the early embryo stages ($7^{th}$, $11^{th}$, $15^{th}$, and $17^{th}$ day embryos, Fig. 5A), as well as in the adult heart, kidneys, brain, muscle, testis, and liver (Fig. 5A).

Northern blot hybridization of poly A+ RNA from three different mouse strains with *Cd59a* and *Cd59b* specific probes revealed different patterns of expression of *Cd59a* and *Cd59b*. After one day of autoradiography, a 1,500-nucleotide band was detected in cardiac, brain, and renal tissues with the *Cd59a*-specific probe, but not with the *Cd59b*-specific probe (data not shown). Exposure of the same membrane for 5 days revealed well-defined 500, 600, and 1,500-nucleotide *Cd59a* bands in the same tissues. In contrast, with a *Cd59b*-specific probe, a stronger 500-nucleotide band and a

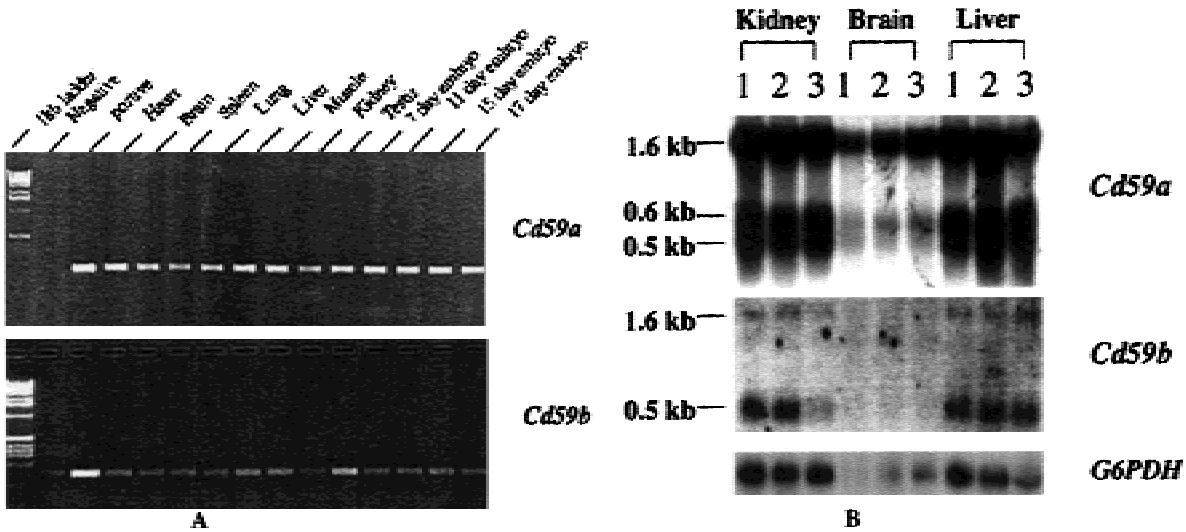


**Fig. 5.** Tissue distribution of mouse *Cd59a* and *Cd59b* mRNA. **A.** A mouse multiple tissue cDNA panel was screened by PCR by using *Cd59a* and *Cd59b* specific primer pairs. Negative (no template) control, positive control (*Cd59a* and *Cd59b* plasmids), and a cDNA panel were amplified by PCR. **B.** Northern blot analysis of poly A+ RNA from three different strains (1: 129/sv; 2: Cast/Ei; 3: C57BL/BJ) with *Cd59a* and *Cd59b*-specific probes. The membrane was first hybridized with a *Cd59b* cDNA specific probe. The membrane was then stripped and rehybridized with *Cd59a* cDNA specific probe. The top picture shows the *Cd59a* expression pattern after autoradiograghy for 5 days; the middle picture shows the *Cd59b* expression pattern after autoradiography for 5 days; and the bottom picture shows G6PDH expression as a control for the poly A+ RNA loading.

weaker 1,500-nucleotide band were observed only after a 5-day exposure. Thus, the predominant *Cd59a* transcript is 1,500 nucleotides long, while the predominant *Cd59b* transcript is 500 nucleotides long. These results confirmed the specificity of the probes used and also documented that both *Cd59a* and *Cd59b* mRNAs are expressed in the kidney, brain, and heart tissues of different mouse strains. In these three tissues, the expression level of *Cd59a* is higher than that of *Cd59b* (Fig. 5B). These data indicate that there are indeed two *Cd59* genes in mice, regardless of the mouse strain analyzed.

*Tissue distribution of Cd59a and Cd59b protein.* Polyclonal antibodies, specific to Cd59a and Cd59b, were generated to analyze the tissue distribution of these gene products. Since the amino acid sequence of Cd59a and Cd59b is 63% identical, the poorly conserved regions in the predicted active site of each protein were selected for immunization: QRCWKQSDAHGEIIMDQLEETKLKF for Cd59a and QQCWKLSDANSNYIMSRLDVAGIQS for Cd59b. Antibody titer and specificity were first determined by Western blot analysis by using extracts of CHO cells expressing either FLAG-Cd59a or FLAG-Cd59b proteins. Briefly, the extracts were separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with anti-FLAG antibodies, preimmune or immune rabbit serum. Specific recognition of Cd59a or Cd59b by the respective antibody was clearly observed at immune sera dilutions as high as 1:800 (data not shown). The specificity of the antibodies was also confirmed by FACS analysis. CHO cells expressing FLAG-Cd59a or FLAG-Cd59b protein were exposed to pre- or immune serum as well as to affinity-purified antibodies. The results show that our anti-Cd59a antibodies recognized cells expressing Cd59a, but not cells expressing Cd59b. Conversely, anti-Cd59b antibodies recognized only the cells expressing Cd59b (Fig. 6).

Immunohistochemistry analysis of mouse tissue with specific anti-Cd59a and anti-Cd59b antibodies revealed that both proteins are expressed in renal glomeruli, soma, and dendrites of cerebral neurons, macrophages in splenic red pulp, bronchial epithelia, testis, and blood vessels of all the tissues screened. Interestingly, the testicular specimens showed that Cd59a is expressed only in spermatids, whereas Cd59b is expressed only in mature sperm (Fig. 7). In addition, both proteins appeared to be diffusely and uniformly expressed in all tissues of a 12-day embryo, and in the muscle, liver, and placenta of adult mice (data not shown).

## Discussion

We have characterized the genomic structure of the two genes encoding the complement regulatory protein CD59 in mouse genome (Fig. 1). These genes, known as *Cd59a* and *Cd59b*, span 45.6 kb on mouse Chr 2 (Fig. 3), where the 15-kb *Cd59b* gene is located 11.6 kb upstream from the 19-kb *Cd59a* gene. While this work was in progress, Holt et al. reported a 13-kb genomic sequence covering *Cd59a* exons 2, 3, and 4 (Holt et al. 2000). This sequence is 99.2% identical to the corresponding region of our *Cd59a* sequence. *Cd59a* and *Cd59b* genes each contain four exons and are approximately 60% identical at the genomic level, 83% at the mRNA level, and 60% at the protein level (Qian et al. 2000). Southern blot comparison of human and mouse genomic DNA with human- and mouse-specific *Cd59* probes confirmed that there is only one *CD59* gene in the human genome, as previously indicated by genomic sequence analysis (Petranka et al. 1992; Qian et al. 2000; Tone et al. 1992; Bickmore et al. 1993). Similarly, the

**Fig. 6.** Characterization of mouse Cd59a and Cd59b antibodies. **A.** CHO cells expressing FLAG-Cd59a (top) or FLAG-Cd59b (bottom) proteins were stained with preimmune-serum (black), Cd59*a* immune-serum (red), or affinity-purified anti-Cd59a antibodies (green). **B.** CHO cells expressing FLAG-Cd59a (top) or FLAG-Cd59b (bottom) were stained with preimmune-serum (black), Cd59b immune-serum (red), or affinity-purified anti-Cd59b antibodies (green).

**Fig. 7.** Tissue distribution of Cd59a and Cd59b. Brain, kidney, testis, spleen, and lung sections were incubated with purified anti-Cd59a and Cd59b antibodies, washed extensively, and then incubated with HRP conjugated secondary antibody. Staining with secondary antibodies alone was used as negative control.

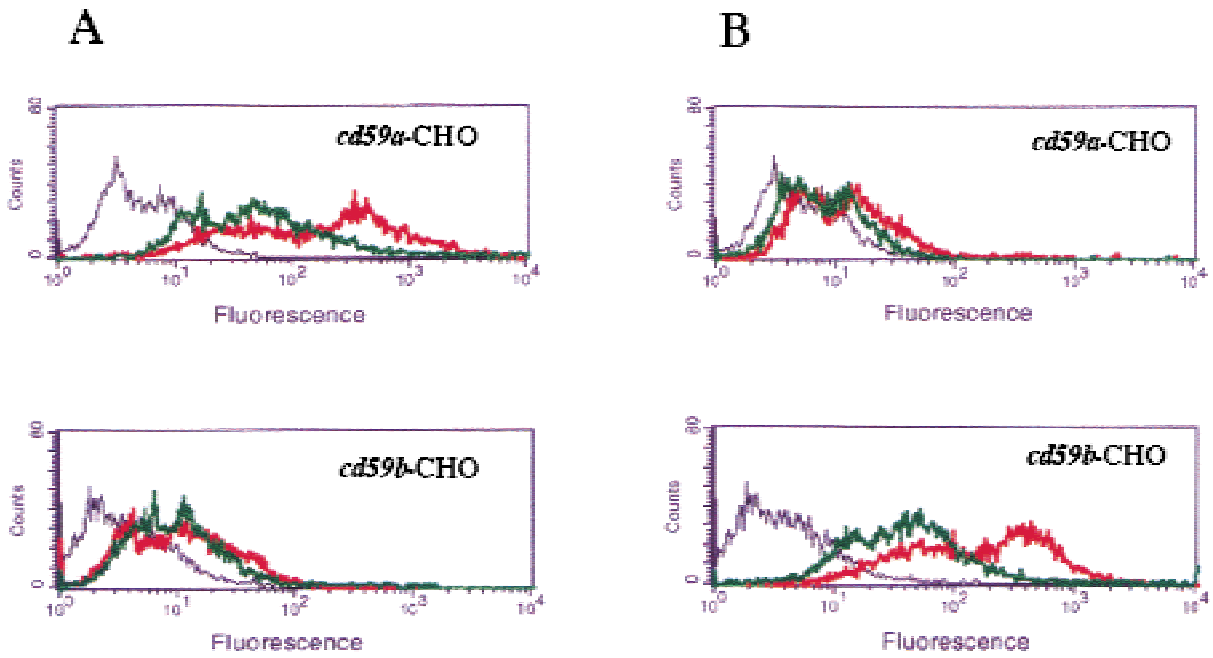
A
B
cd59a-CHO
cd59a-CHO
cd59b-CHO
cd59b-CHO
Counts
Fluorescence

**Fig. 6.**

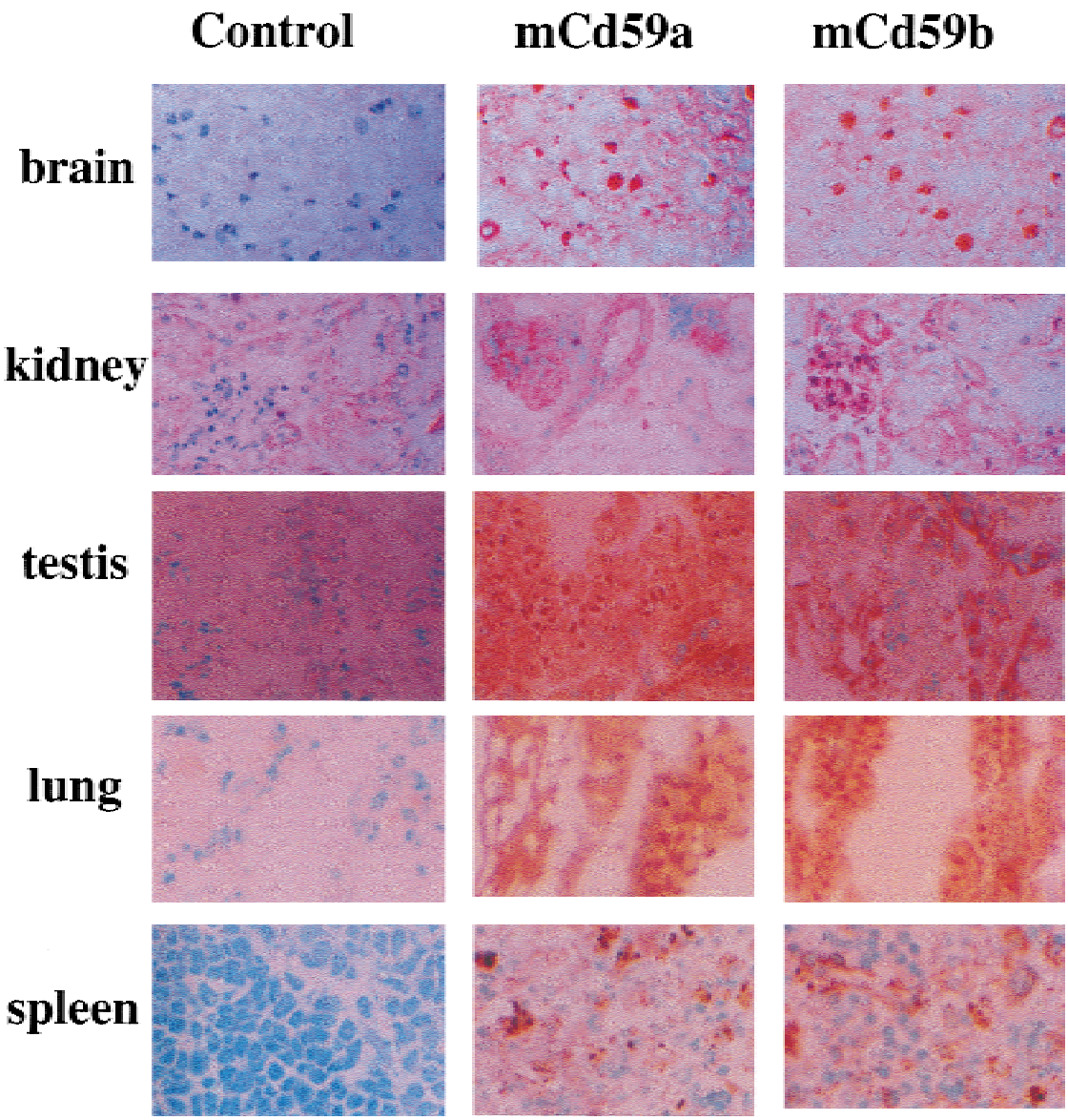
Control
mCd59a
mCd59b
brain
kidney
testis
lung
spleen

**Fig. 7.**

human genome contains a single gene for decay accelerating factor (DAF), but the mouse genome contains two *Daf* genes (Spicer et al. 1995; Fukuoka et al. 1996; Song et al. 1996). This peculiar genetic difference of mouse *Daf* and *Cd59* appears to be the result of gene duplication rather than loss of one copy in the human genome. This interpretation is supported by our finding of transposon repeat sequences in the mouse *Cd59* genomic structure. A 5.0-kb L1 retro-transposon repeat sequence is located between exons 1 and 2 of *Cd59b*, and another 3.5-kb mouse early transposon sequence lies between *Cd59a* and *Cd59b* (Fig. 1). There are approximately 3,000 copies of the L1 retrotransposon in the mouse genome, a number 75 times greater than that in the human genome (DeBeradinis et al. 1998). The considerable difference in the frequency of this repeat may contribute to ongoing gene evolution, since retrotransposition is believed to increase the probability of gene duplication and to generate insertional mutations that cause different genetic diseases (Kazazian and Moran 1998). Thus, it is possible that retrotransposons were responsible for the gene duplication events that resulted in two *Cd59* genes in the mouse genome. Given the major role of complement in physiology and pathophysiology, it is possible that the presence of two *Daf* and *Cd59* genes in the mouse genome confers some form of genetic advantage that favors the preservation of two genes for each one of these complement regulators.

The human CD59 gene has four exons and spans 26 kb (Petranka et al. 1992; Qian et al. 2000). The genomic organization of human CD59 is similar to that of each of the mouse *Cd59* genes (Fig. 1). This suggests that the CD59 gene may be conserved among different species at the genomic level. It has recently been reported that the human CD59 gene contains a previously unknown 5$^{th}$ exon between exons 1 and 2, and that 10–20% of the human CD59 mRNA contains the 5$^{th}$ exon in the 5′ untranslated region (Holguin et al. 1996). We have found that a sequence identical to exon 1 of *Cd59b* is present between exons 1 and 2 of *Cd59a*. Whether this additional exon is transcribed into mRNA is not yet known. If mouse *Cd59* also contains this novel exon in a fraction of its mRNA, the *Cd59* gene would be very conserved between human and mouse; such a conserved gene structure usually reflects an important biological function of the encoded protein. Alternative exons in the 5′UTR of *Cd59* mRNA may play a role in its translational regulation.

The tissue expression of Cd59a has been analyzed previously (Powell et al. 1997). Since Cd59b was not known at that time, the antibody used may have recognized both mouse Cd59 proteins. Using anti-Cd59a and anti-Cd59b-specific antibodies, we analyzed the tissue expression of both proteins and showed that both are widely expressed in most organs (Fig. 7). In the testis, Cd59a is expressed exclusively in the spermatids, whereas Cd59b is expressed in more mature sperm cells (Fig. 7). The striking difference in the pattern of testicular Cd59 expression confirms the specificity of the antibodies used, and suggests a possible specific role for Cd59a and Cd59b in reproduction. Indeed, complement regulatory proteins expressed in human reproductive organs and gametes are thought to play a pivotal role in protection against autologous complement activation in the genital tract (He et al. 2000).

We have recently proposed that glycation-inactivation of human CD59 contributes to the development of vascular complications of diabetes (Acosta et al. 2000). Consistently, immunocytochemical analysis of several complement proteins has shown focal complement activation and MAC deposition in endoneurial microvessels of patients with diabetic neuropathy but not in patients with other neuropathies (Rosoklija et al. 2000). Glycation of human CD59 is due to the presence of a glycation motif K41-H44 in its active site (Acosta et al. 2000). This glycation motif is not present in the CD59 from any other species sequenced to date. We have postulated that the presence of the glycation motif in human CD59 might explain, in part, the peculiar propensity of humans to develop the vascular complications associated with diabetes. It is now imperative to develop animal models to test this hypothesis. The extensive study of the mouse *Cd59* genomic structure, functional comparison, and tissue distribution reported here will aid in the development of molecularly engineered mice carrying a humanized CD59 protein containing the H44 residues required to glycate K41. If these animal models develop diabetic vascular complications in a manner comparable to the human disease, they will become an important tool in studying the pathophysiology of diabetes. Moreover, these mouse models will provide an invaluable tool for testing novel therapeutic approaches for a disease that represents a major cause of morbidity and mortality in the human population.

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