

Article

Activity after Site-Directed Mutagenesis of CD59 on Complement-Mediated Cytolysis

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CD59 may inhibit the cytolytic activity of complement by binding to C8/C9 and protect host cell membranes against homologous membrane attack complex (MAC). However, CD59 is widely overexpressed on tumor cells, which has been implicated in tumorigenesis. The active site of CD59 relative to MAC is still confused. As reported the MAC binding site is located in the vicinity of a hydrophobic groove on the membrane distal face of the protein centered around residue W40. Here two site-directed mutagenesis were performed by overlapping extension PCR to delete residue W40 site (Mutant 1, M1) or to change C39W40K41 to W39W40W41 (Mutant 2, M2). Then we constructed mutant CD59 eukaryotic expression system and investigated their biological function on CHO cells compared with wild-type CD59. Stable populations of CHO cells expressing recombinant proteins were screened by immunotechnique. After 30 passages culturing, proteins could be tested. Dye release assays suggest that M1CD59 loses the activity against complement, while M2CD59 increases the anti-complement activity slightly. Results indicate that W40 of human CD59 is important to its activity, and prohibition of this site may be a potential way to increase complement activity and to treat tumors. *Cellular & Molecular Immunology*. 2008; 5(2):141-146.

Key Words: CD59, complement, active site, site-directed mutagenesis, overlap extension PCR

Introduction

Complement is a tightly regulated system of proteins that protects hosts from infection by microorganisms or the growth of tumors. Complement-mediated immune responses act in the assembly of MAC at the membrane of the cell, forming a pore that leads to osmotic lysis (1, 2). MAC is formed by the self assembly of C5b, C6, C7, C8, and multiple C9 molecules. In order to limit damage to host tissues, it is therefore essential to strictly control the activation. CD59 was first identified as a regulator of the terminal of complement, which acted by binding to the C8/C9 components, thus preventing the formation of MAC, to interfere with C9 membrane insertion, polymerization and pore formation (3-5). The precursor of human CD59 is a

single peptide composed of 128 amino acids deduced from its cDNA sequence (6, 7). The mature protein consists of 77 amino acids arranging in a single cysteine-rich domain composed of two antiparallel β -sheets, 5 protruding surface loops and a short helix (8-10).

CD59 is widely distributed in various cell membranes, protecting host cells from the cytolytic action of MAC. CD59 expression has also been implicated in tumorigenesis and in providing cancer cells with protection from monoclonal antibody immunotherapy (11-13). Thus, knowledge of the CD59 binding interface may also assist with the rational design of molecules that inhibit CD59 function and enhance cancer cell susceptibility to antibody therapy. Previous studies of the CD59 binding interface indicated that residues 1-41 inhibited the MAC formation much more strongly than 42-77 (14), and its C8 (C5b-8) and/or C9 (C5b-9) binding site was located in the vicinity of a hydrophobic groove on the membrane distal face of the protein centered around residue W40 and closed to the helix (15-17).

Our previous studies have investigated the functional activity of the vicinity around W40 through mutation on N37H, K38H, F42K, F42H, E43H, H44Q, and N46H. The results demonstrated that CD59 mutants still had its activities (18-20) and N37, K38, F42, E43, H44, N46 are probably not the active sites. In this study we addressed the biological relevance of the CD59 W40 site and its vicinity by examining the biochemical, biophysical, and functional properties of two mutants of CD59 missing the highly

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conserved W40 site or changing C39W40K41 to W39W40W41.

Materials and Methods

Materials

Human CD59 cDNA plasmids were obtained from Dr. Halperin (Harvard Medical School, Boston, MA, USA). Chinese hamster ovary (CHO) cell was purchased from Chinese Academy of Science. RPMI-1640 was purchased from GIBCO (Gaithersburg, MD, USA); Trypsin and G418 antibiotic were from Amersco; Lipofectamine 2000 was from Invitrogen TM Life Technologies. PMD18-T vector, eukaryotic expression pIRES plasmid, *EcoR* I, T4 DNA ligase, marker DL2000 were from Takara (Japan), and *E. coli* JM109 was conserved in our lab. The anti-CD59 antibody SC7077 was purchased from Santa Cruz Biotechnology; the polyclonal antisera specific for CHO cell membranes were prepared in house using standard methods. FITC-conjugated CD59 fluorescence antibody and FITC-conjugated goat anti-mouse IgG were purchased from COULTER.

Selection of mutant genes

According to CD59 NMR configuration and to the previous studies, W40 site and C39W40K41 gene were chosen for mutant (Table 1).

Mutagenesis

Primers were designed as Table 2 shows, pT7 (upstream) and T3 (downstream) are normal primers, M1F (upstream) and M1R (downstream) are mutagenesis primers which delete the TGG encoding W40 at the center region; M2F (upstream) and M2R (downstream) are primers changing C39W40K41 to W39W40W41 of CD59.

Reconstruction of CD59 by overlap extension PCR

To obtain the mutant CD59, we employed the site-directed mutagenesis to delete residue W40 or change C39W40K41 to W39W40W41. Together with common primer pT7 and T3, overlap extension PCR was performed. pT7 and M1R (or M2R) were used as primers for PCR1: predenaturation at 94°C for 3 min; 25 cycles with denaturation at 94°C for 30 s, annealing at 42°C for 45 s and elongation at 72°C for 45 s; finally full elongation at 72°C for 7 min. M1F (or M2F) and T3 were used as primers for PCR2: the amplification condition were same as PCR1, and templates for PCR1 and PCR2 were the human CD59 cDNA plasmids. For PCR3, templates were products of PCR1 and PCR2 purified by

DNA Gel Extraction Kit (Bio-Dev, China) and mixed at equal mole. pT7 and T3 as primers for PCR3: predenaturation at 94°C for 3 min; 25 cycles with denaturation at 94°C for 30 s, annealing at 46°C for 45 s and elongation at 72°C for 45 s; finally full elongation at 72°C for 7 min. The PCR3 products were isolated by electrophoresis in a 1% agarose gel in the presence of 0.5% ethidium bromide and the amplification of about 650 bp that encodes mutant human CD59 (M1CD59 or M2CD59) was purified with DNA Gel Extraction Kit.

Construction of cloning recombinant vector

The length of cloning vector PMD18-T-vector is 2,692 bp containing ampicillin antibiotic resistance gene. Purified PCR products M1CD59 or M2CD59 were ligated with PMD18-T-vector by T4 ligase at 16°C overnight. Then recombinant clone was transformed into *E. coli* JM109 by CaCl₂ method, and plasmids were extracted with alkaline lysis method.

Acquisition of eukaryotic expression plasmid pIRES

pIRES plasmids containing G418 antibiotic resistance gene were transformed into *E. coli* JM109 strain, then were amplified and extracted. The length of the eukaryotic expression vector pIRES is 6.1 kb and contain *EcoR* I restriction site, ampicillin and G418 antibiotic resistance gene.

Construction of expression recombinant vector

The mutant PMD18-T-M1CD59-vector was digested by *EcoR* I, fragment containing the translated region of a CD59 complementary DNA was subcloned into the lined and dephosphorylated expression vector pIRES. The recombinant pIRES-M1CD59-vector was identified by PCR and enzyme digestion. Positive clones were sequenced (Takara). The same process was employed for M2CD59 and pIRES-M2CD59-vector construction.

Screening of stable positive cell clones by CD59 mRNA by *in situ* hybridization

Corresponding to CD59 mRNA, a 27 bp oligonucleotide probe labeled with digoxin at 5' end (5'-CTC ATT ACC AAA GCT GGG TTA CAA GTG-3') was synthesized by Bioasia Biotechnology Company. Transfected CHO cells were dropped on microscope slides disposed by 0.1% poly-lysine solution and fixed at room temperature for 20-30 min by 4%

Table 1. Mutant and wild type human CD59

Protein	37	38	39	40	41	42	43	44	45	46
Wild	N	K	C	W	K	F	E	H	C	N
M1	N	K	C	-	K	F	E	H	C	N
M2	N	K	W	W	W	F	E	H	C	N

Table 2. Primers for mutagenesis on CD59 gene

Primer	Primer sequence (5'→3')
pT7	TAATACGACTCACTATAGGC
T3	ATTAACCCTCACTAAAGGGA
M1F	AGTGTATAACAAGTGTAAGTTTGAGCA
M1R	TGCTCAAACCTACTTGTATACACT
M2F	GTGTATAACAAGTGGTGGTGGTTTGAGCATTGC
M2R	ATGCTCAAACCACCACCACTTGTATACACTTG

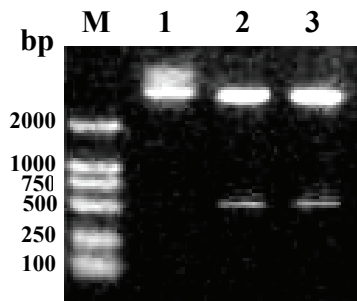


Figure 1. Identification of recombinant plasmids. Electrophoresis in a 1% agarose gel in the presence of 0.5% ethidium bromide. Lane M, DL2000 DNA marker; Lane 1, pIRES-M1CD59 vector; Lane 2, pIRES-M1CD59 plasmid digested by *EcoR* I, M1CD59 was about 500 bp; Lane 3, pIRES-M2CD59 digested by *EcoR* I, M2CD59 was about 500 bp.

polyformaldehyde 0.1 mol/L PBS (pH 7.2-7.4), washed and treated with 30% H₂O₂ methanol (1:50) at room temperature for 30 min to block endogenous peroxidase activities and then washed with distilled water. Prehybridization was performed by adding 20 μ l prehybridizational liquid on microscope slide and incubated in wet boxes at 38-42°C for 2-4 h. Then 40 μ l of hybridizational liquid containing 40 ng probes was added on the cells which were then incubated overnight and washed with SSC. Because of digoxin, the CD59 mRNA expression could be determined by immunohistochemical staining. In brief, the cells were incubated in blocking buffer followed by the biotinylated polyclonal antimouse IgG, ABC and DAB and the results were observed under light microscope. After the positive CHO cell clones with CD59 gene were screened and ampliatively cultured, stable transfected cell lines were established. The controls were transfected with pIRES which lacks CD59 sequence.

Immunological fluorescence

CHO cells stably transfected with pIRES-M1CD59, pIRES-M2CD59, pIRES-WTCD59 and pIRES were washed twice with phosphate-buffered saline (PBS), then were dropped on microscope slides and fixed in 95% alcohol for 5 min respectively. These cells were incubated in FITC-goat anti-human CD59 mAb at 37°C for 45 min, and observed under fluorescence microscope.

Enzyme linked immunosorbent assay (ELISA)

In order to test the expression of CD59, an ELISA assay was established utilizing polyclonal goat-anti-human CD59 as first antibody and peroxidase-conjugated rabbit anti-goat IgG as second antibody. Lysate of protein at a final concentration of 10 μ g/ml diluted duplication in phosphate-buffered saline was incubated in the wells of microtitre plates overnight at 4°C. The plates were washed three times in PBS containing 0.05% Tween 20, and then CD59 PcAb diluted in PBS (1:400) was added. After incubation at 37°C for 1 h the wells were again washed and peroxidase-labelled rabbit anti-goat (IgG) antibody at a final dilution of 1:4,000 in PBS was added.

WT AGTGTATAACAAGTGGTTGGAAGTTTGAGCA
 M1 AGTGTATAACAAGTGT - - - AAGTTTGAGCA
 M2 AGTGTATAACAAGTGGTGGTGGTTTGAGCA

Figure 2. Sequence identification of recombinant plasmids. The recombinant plasmids of WTCD59, M1CD59 and M2CD59 were sequenced in Takara.

After further 1 h at 37°C the plate was washed, peroxidase substrate (1,2-diphenylenediamine dihydrochloride; Pierce) was added and colour allowed to develop for 15 min in a light-avoiding position. The reaction was then stopped with 2 M H₂SO₄ and the A450 in the wells read using a Bio-TEK instruments microplate reader.

Activity tested by dye release assay

Anti-complement activities of M1CD59 and M2CD59, as well as the functional differences between wild-type CD59 and control groups were measured by dye release assay. In brief, CHO cells (2×10^5 /ml, transfected by wild-type CD59, M1CD59, M2CD59 and empty pIRES plasmids) were seeded in 96-well plastic plates. After BCECF fluorescence dye was added, rabbit-anti-CHO antibody and fresh normal human serum with different dilution were used. Normal human serum was obtained from the blood of healthy volunteers in the laboratory. Supernatant and fission product were collected respectively, and were read in a Cary Eclipse fluorimeter with the excitation filter set at 503 nm and emission filter at 530 nm. The remaining cells were lysed with 0.05 ml PBS containing 0.1% Triton X-100 during 30 min incubation at room temperature, then the lysate for release assay was read again. Mean and standard deviations were determined from triplicate samples. % lysis = [supernatants fluorescence release / (supernatants fluorescence release + detergent fluorescence release)] \times 100.

Statistical analysis

Student's *t* test was used to analyze the data. Data were presented as the mean \pm SD. *p* values of < 0.05 were considered statistically significant.

Results

Construction and identification of expression vector

PCR products that encode mutant human CD59 (M1CD59 or M2CD59) were about 650 bp. The products were digested and ligated with PMD18-T-vector to construct clone recombinant vectors. Then the clone recombinant vectors PMD18-T-M1CD59-vector and PMD18-T-M2CD59-vector were digested with *EcoR* I. Digested M1CD59 and M2CD59 were about 500 bp. Then the digested products were extracted and purified from gel, ligated with lined and dephosphorylated pIRES vector, identified by PCR, *EcoR* I digesting (Figure 1) and sequenced in Takara (Figure 2).

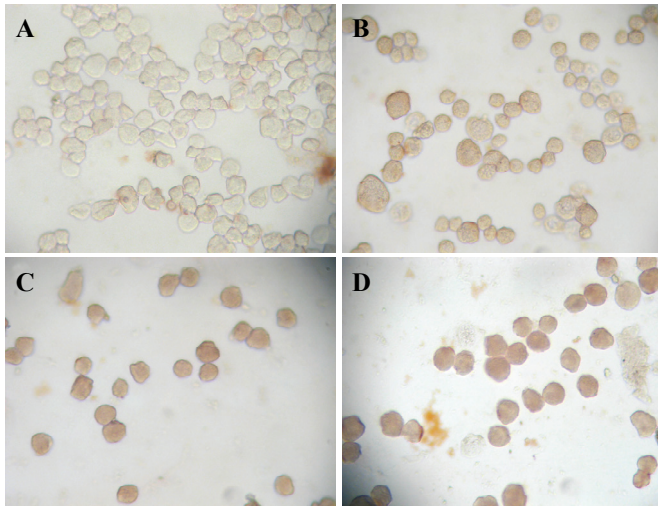


Figure 3. CD59 mRNA expression in transfected cells detected by *in situ* hybridization. (A) CHO cells transfected with pIRES; (B) CHO cells transfected with pIRES-WTCD59; (C) CHO cells transfected with pIRES-M1CD59; (D) CHO cells transfected with pIRES-M2CD59.

Screening of stable transfected cell clones by CD59 mRNA nucleic acid by *in situ* hybridization

Synthesized CD59 oligonucleotide probe was applied to determine the expression of CD59 mRNA gene. The results of *in situ* nucleic acid hybridization observed by optical microscope showed that positive cell clones, namely pIRES-WTCD59, pIRES-M1CD59 and pIRES-M2CD59 transfected CHO cells were stained brownish yellow, and the dying site resided in cytomembrane and cytoplasm. While

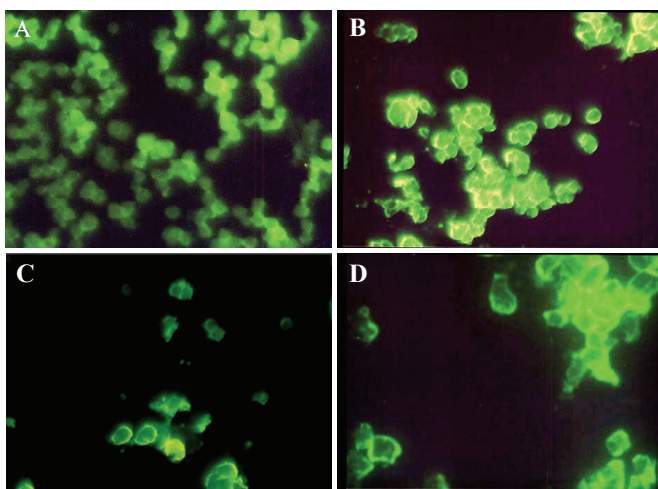


Figure 4. Cell surface CD59 expression by immunological fluorescence assay. (A) CHO cells transfected with pIRES (200 \times), (B) CHO cells transfected with pIRES-WTCD59 (200 \times), (C) CHO cells transfected with pIRES-M1CD59 (200 \times), (D) CHO cells transfected with pIRES-M2CD59 (200 \times).

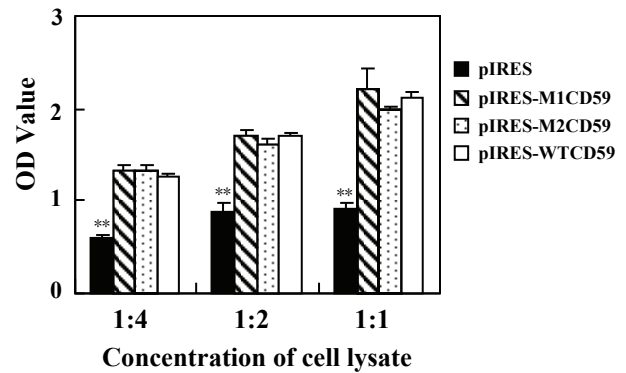


Figure 5. Detection of CD59 protein expression in the lysates of transfected CHO cells. The lysates of various transfected CHO cells were obtained and the CD59 expressions were detected by ELISA as described in the Materials and Methods. ** $p < 0.01$, compared with pIRES-M1CD59, pIRES-M2CD59 and pIRES-WTCD59 groups.

the control groups pIRES transfected CHO cells had no positive expression and the staining was hypochromic (Figure 3).

Identification of CD59 expression with immunological fluorescence assay

To determine the human mutant M1CD59, M2CD59 and wild-type CD59 expressions on CHO cells, we conducted immunological fluorescence with the known CD59 mAb labeled by FITC. The expressions of M1CD59, M2CD59 and wild-type CD59 transfected groups were significantly higher than those of the control group (Figure 4).

Measurement of the quantity of CD59 expression by ELISA

In CHO cell groups with recombinant plasmids pIRES-M1CD59, pIRES-M2CD59, and pIRES-WTCD59, the quantities of CD59 expressions were significantly higher than those of control group CHO cells with plasmid pIRES (Figure 5).

Functional activity detected by dye-release assay

Before exposed to human complement, the target CHO cells were incubated with complement inactivated rabbit-anti-CHO antibody to measure its sensitivity to MAC-mediated cytolysis. As shown in Figure 6, M1CD59 and wild-type CD59 had obvious difference in anti-MAC activity, but no difference between M1CD59 and pIRES control group; while M2CD59 had no significant difference with WTCD59, although its anti-complement activities was slightly higher than that of WTCD59. Results indicated that function loss of M1CD59 was significant and W40 site of CD59 was very important.

Discussion

Human CD59 is widely expressed in blood cells and many

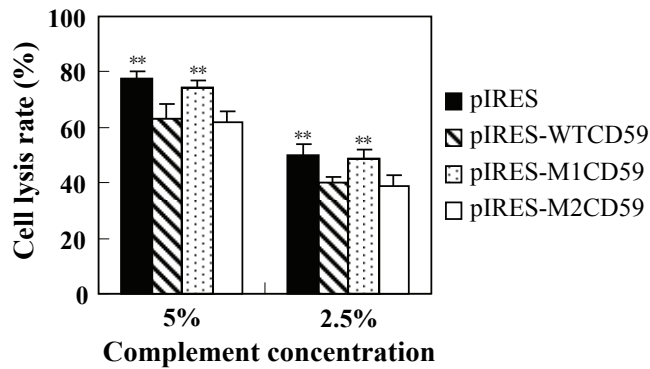


Figure 6. The function activity of mutant CD59 detected by dye release assay. CHO cells (2×10^5 /ml, transfected by wild-type CD59, M1CD59, M2CD59 and empty pIRES plasmids) were seeded in 96-well plastic plates. BCECF (2 μ g/ml), rabbit-anti-CHO antibody and fresh normal human serum containing complement in 5% and 2.5% dilution were added and incubated, then the supernatant and fission product were read in a Cary Eclipse fluorimeter with the excitation filter set at 503 nm and emission filter at 530 nm. ** $p < 0.01$, compared with WTCD59 and M2CD59 groups.

other tissue cells, protecting host cell membranes from homologous MAC. However, CD59 is sometimes over-expressed on tumor cells, and its expression has been linked to promoting tumor growth and the protection of tumor cells from mAb therapy (21). Bjorge L et al. (22) found that CD59 was stably expressed in ovarian cancer throughout the cell cycle, and suggested that the activities of intrinsic C regulators should be neutralized to make minimal residual disease a promising target for antibody therapy. In another study, incubation of epithelial ovarian cancer cells with neutralizing mAbs to CD59 led to a significant increase in the C-dependent cytotoxicity (CDC) from 10%-20% to 45%-50% (23). It is therefore of interest to understand the molecular interaction between CD59 and its complement ligands for designing CD59 inhibitory molecules to enhance tumor cell susceptibility to antibody induced complement cytotoxicity.

From a combination of molecular modeling and mutagenesis techniques, which together indicate that the active site of CD59 is located in the vicinity of a hydrophobic groove on the face of the molecule (16). The sidechain of W40 partially fills a cleft formed by the packing of the α -helix against the 3-stranded β -sheet on the glycosylated face of CD59 (24). Regarding the important W40 site of CD59, we first had two kinds of mutation of deleting the W40 site of CD59 and substituted C39W40K41 to W39W40W41. Interestingly we found that the mutant proteins were stably expressed on CHO cell surface. After 30 passage culture, protein could be tested. Mapping of mutations associated with enhanced or reduced inhibitory function of CD59 showed the binding region to be located in a crevice between $\alpha 1$ and $\alpha 3$ -stranded β -sheet (Figure 7).

Regarding the function of CD59 mutants, dye release

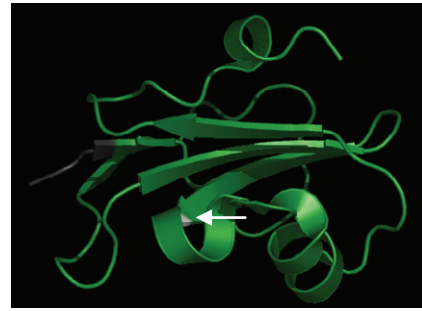


Figure 7. Feature Map 3D - query result of M1CD59. W40 of CD59 is located in the white area as white arrow indicates (from <http://www.cbs.dtu.dk/>).

assays suggest that the mutant CD59 deleted W40 loses its function to inhibit cytotoxic activity of complement, which further confirm the importance of W40 site of CD59. As for the mutant of substituted C39W40K41 to W39W40W41, CD59 does not lose its activity with C39W and K41W mutants, indicating that C39 and K41 are not important for functional activity, therefore further shows that the inhibiting activity depends on the W40 site. From previous studies on the mutagenesis of residues N37-N46, we found that CD59 anticomplement activity was still preserved in CHO cells transfected with these CD59 mutants. Thus W40 of human CD59 is important to its activity, and prohibition of this site may be a potential way to increase complement activity and to treat tumors. Accordingly, we may design polypeptide binding to CD59 including the W40 site, or design imitating molecules binding to C8/C9 to inhibit the CD59 binding to complement, and to increase the complement-mediated anti-tumor effect.

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