J. Biochem. 2009;145(4)533–542
doi:10.1093/jb/mvp006

Construction of the Plasmid, Expression by Chinese Hamster Ovary Cell, Purification and Characterization of the First Three Short Consensus Repeat Modules of Human Complement Receptor Type 1

Atsushi Yamaguchi[1,2,*], Hiroaki Takagawa[1,2,*], Hirofumi Iwakaji[2], Shuji Miyagawa[3], Pi-Chao Wang[1,2,†] and Noriyuki Ishii[1,‡]

[1]Biological Information Research Center, National Institute of Advanced Industrial Science and Technology (AIST), Central-6, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566; [2]Gradute School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572; and [3]Division of Organ Transplantation, Department of Molecular Therapeutics, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871 Japan

Received November 20, 2008; accepted January 7, 2009; published online January 17, 2009

Short consensus repeat (SCR1-3), the first three SCR modules from N-terminus of type 1 complement receptor (CR1), is expected to accelerate dissociation of complement components and suppress complement activity by binding the main component of complement C4b. In order to clarify the three-dimensional structure, which triggers the activity of SCR1-3 on complement, we constructed an over-expression system in CHO DG44 cells which facilitated mass production of SCR1-3. The mass production was achieved by a two-stage culture system and optimum culture conditions using ASF104N medium and MTX-, NaBu-containing α-MEM/10% FBS medium, respectively. The constructed gene of SCR1-3 was confirmed by restriction enzyme digestion and DNA sequence analysis, and the expressed protein by CHO DG44 cells was confirmed by western blotting. The expressed SCR1-3 was proved containing N-linked sugar chain, an important factor to the proper expression of protein, by the cleavage with glycosidase of N-linked oligosaccharide (PNGase F). The suppression effect of the yield protein on complement-mediated inflammation was investigated by haemolytic assay and necrosis assay of stromal cells. Both assays showed that SCR1-3 possessed complement control activity. However, residing sugar chain on SCR1-3 did not show significant difference in the complement control activity.

Key words: complement control activity, complement receptor type 1 (CR1), short consensus repeat (SCR1-3), CHO cell, sugar chain.

Abbreviations: CHO, Chinese hamster ovary; CR1, complement receptor type 1; DHFR, dihydrofolate reductase; dhfr, dihydrofolate reductase gene; MAC, membrane attack complex; MTX, methotrexate; NaBu, sodium butylate; NHS, normal human serum; NMR, nuclear magnetic resonance; PNGase F, peptide-N4-(acetyl-β-glucosaminyl)-asparagine amidase; SCR, short consensus repeat; sCR1, soluble form of CR1; SCR1-3, the first three SCR modules from N-terminus of CR1; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SRBC, sheep red blood cells.

The complement system in human is sequential complex arrays of interacting protein reactions involving in a number of physiological events. A variety of physiological responses, such as increasing of vascular permeability, chemotaxis of phagocytic cells, activation of inflammatory cells, opsonization of foreign invasions, cell lysis, and damage on tissues in pathological state, are caused by the activation of complement system. Complement receptor type 1 (CR1, CD35) is a membrane glycoprotein that plays a role as a regulator of complement activation in controlling many events associated with the immune responses. For example, CR1 is found expressed on erythrocytes (*1*), a subset of T cells, mature B cells, glomerular podocytes, monocytes, granulocytes and splenic follicular dendritic cells (*2*), and can prevent the complement-mediated red cell destruction (*3*). Moreover, the number of CR1 in erythrocyte was found drastically decreased in patient with systemic lupus erythematosus (*4*), and the soluble form of CR1 (sCR1) was also found effective to suppress the post-ischaemic myocardial inflammation and necrosis (*5*).

The molecular mass of CR1 is 160–250 kDa depending on its allotypes, and the extra-cellular portion of the

*These authors are equal contributors to this work.
†To whom correspondence should be addressed. Tel: +81 29 853 7098, Fax: +81 29 853 7098,
E-mail: cogitate@sakura.cc.tsukuba.ac.jp
Correspondence may also be addressed to Noriyuki Ishii. Tel: +81 29 861 6195, Fax: +81 29 861 6195,
E-mail: ishii@ni.aist.go.jp
‡Present address: Institute for Biological Resources and Functions, National Institute of Advanced Industrial Science and Technology (AIST), Central-6, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan.

most common allotype comprises 30 modules known as a series of tandemly arranged short consensus repeat (SCR). Each SCR contains 60 to 70 amino acids, and 45% of the average 65 residues are conserved in each SCR. Four conserved cysteine residues, the first and third, and the second and fourth cysteines are covalently linked through disulfide bonds, are seen in each SCR commonly (*6*).

SCR1-3, the first three modules of CR1, binds to C4b, which is a site to activate the decay acceleration of classical and alternative pathways of C3 convertase. In the decay acceleration activity, CR1 plays a role as a complement inhibitor by reversibly binding to C3b and C4b, and inactivating the C3, C5 convertase and the protein complex consisting of C3b and C4b (*7*, *8*).

Rising attention has been paid to CR1 due to its capability of inhibiting the inflammatory reaction in complement system. The crystal structures of complement components C2a, C3b have been analysed, which provide insights into complement activation and regulation (*9–11*). To elucidate the function of complement regulation at the molecular level, the identification of the three-dimensional structure of CR1 is important. A structural model of soluble form of CR1 (sCR1) derived from the combination of small angle X-ray scattering and analytical centrifugation has been reported recently (*12*). However, in the reported model, only the solution structure for SCR module determined by nuclear magnetic resonance (NMR) is arranged as a repetitive unit (*13*), and further investigation on the insight to SCR structure is still remained. To achieve the detailed structural determination at atomic resolution by the X-ray diffraction method, construction of a supplying system with plenty of the small target protein, for example SCR1-3, is necessary.

In this study, we constructed a plasmid which could effectively express SCR1-3 in CHO cells by amplifying the target gene, and the positive clones of SCR1-3 expressing cells could be easily screened. Moreover, the expressed protein can be recovered in the supernatant of cell culture by means of a two-stage cell culture method (*14*). We also confirmed the inhibition activity of the purified protein on the complement-mediated inflammation.

CR1 is a glycoprotein. In general, glyco-chains are thought as a requisite for proper expression of protein functions, thus CHO cell was employed as a host by which post-translational glyco-modification was allowed to occur. According to the investigation on the glycosylation of sCR1, this receptor is comprised of a single polypeptide chain and 25 sites have been proposed, 14 of which are experimentally known to be occupied by N-linked complex oligosaccharides and no O-linked carbohydrates exist (*15*). Examination of the homology in the sequences of SCR structures and the two solution structures determined by NMR for CR1 SCR15-16, and SCR16-17 show that all but one of these 25 putative sites are located close to the centre of SCR domain instead of the inter-SCR linking sites (*13*). We characterized SCR1-3 by investigating the functional role of sugar-chains, and the results showed that the sugar chains on SCR1-3 did not influence the inhibition activity on the complement-mediated inflammation. These information obtained in related to the role of sugar-chains on SCR1-3 should approve crystallization and structure analysis by X-ray diffraction method of SCR1-3 which was also prepared from non-glycosylation systems because SCR1-3 as it was assumed to fold in similar manner.

MATERIALS AND METHODS

Construction of Plasmid pCX-N2L-SCR1-3—The cDNA that encodes SCR1-3 of human CR1 connecting the secretion signal sequence was cloned to the animal cell expression vector pCX-N2L. For the convenient selection and gene amplification with methotrexate (MTX) (*16*), dihydrofolate reductase (dhfr) gene was introduced into the plasmid by the following methods: The SV40 promoter and the dhfr gene were cut out from pSV2-dhfr (ATCC: 37146) (*17*) by using restriction enzymes, *Pvu*II and *Pst*I. Both termini of the fragment which contained SV40 promoter and SV40 poly(A) signal were smoothed by Blunting high (Toyobo, Osaka), then the gene fragment was inserted into *Eco*R V site of pBluescript II KS (+/–) which was named as pBluescript-dhfr.

Total RNA of CHO35.6 cell (ATCC: CRL-10052) which could secrete soluble CR1 (sCR1) was extracted by TRIzol Reagent (Invitrogen Corporation, Carlsbad, CA). The RNA was purified by Oligotex^{tm}-dt30 <Super> mRNA Purification Kit (Takara Bio Inc., Shiga) and then converted to cDNA by SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA). These kits were used according to the manufacturer's recommendation.

SCR1-3 gene was amplified by PCR using the oligonucleotides: 5′-TTTCTCGAGTGGGGAGAATGGGGGCCT CTTC-3′ for forward and 5′-TTTGGTACCTTAGTGATG GTGGTGGTGGTGGCTGCCGCGGCCCTCGATAGGTAT AATGCACTGAGGGGC-3′ for reverse. The primers also comprised the sequences of Factor Xa site, 8xHis-tag, stop codon and *Xho*I site at the 3′ end of SCR1-3 gene. This PCR product was inserted into pBluescript II KS (+/–) for cloning. The insert was checked by colony direct PCR. The fragment encoding SCR1-3 was digested with *Sma*I and *Hinc*II and ligated into *Eco*RV site of pCX-N2L which was composed of CMV-IE promoter/enhancer and rabbit β-globin poly(A) signal for high expression in mammalian cells.

The above vectors of pBluescript-dhfr and pCX-N2L-SCR1-3 were digested by *Sal*I and *Spe*I, respectively. The obtained fragment encoding dhfr from pBluescript-dhfr was ligated to pCX-N2L-SCR1-3. This constructed plasmid was confirmed by restriction enzyme digestion and DNA sequencing, and finally named as pCXD-SCR1-3. In cloning for all constructed plasmids, *Escherichia coli* DH5α treated with calcium chloride was used as a competent cell for transformation.

Expression of SCR1-3—The constructed vector was purified by midi prep using Hispeed Plasmid Midi Kit (Qiagen Inc., Valencia, CA), then transfected into CHO DG44 cell using Lipofectamine2000 following the manufacturer's protocol. CHO DG44 cell is known as dhfr-deficient CHO cell, which was cultured in α-MEM with nucleic acid (Gibco/Invitrogen Corporation, Carlsbad, CA) containing 10% FBS beforehand. To select positive clones of interest, transfectants were cultured for 2 weeks in

α-MEM containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin but no nucleic acid was added therein. The medium was changed every 3 days.

In order to increase yield of the expressing product, MTX was added to the medium to induce the response of dhfr gene. The concentration of MTX was increased from 50 to 350 nM, and from 350 to 1,000 nM stepwisely. Colonies with high tolerance were checked at each stage. After cells reached 90% confluency and positive clones were selected by limiting dilution, the desired clone was established. The expression of SCR1-3 was confirmed by RT-PCR and western blot. When CHO DG44 cells reached confluency in 60 mm culture dish, they were trypsinized by PBS containing 0.05% trypsin and 50 mM EDTA, and then were collected for the use in RT-PCR. The related RNA was extracted from the collected cells with High Pure RNA Tissue Kit (Roche Diagnostics K.K., Basel, Switzerland), and the cDNA was obtained *via* reverse transcription by means of ReverScriptIII (Wako Pure Chemicals Industries, Osaka), followed by PCR with primers that were able to read the whole sequence of SCR1-3 (Hokkaido System Science Co., Ltd., Sapporo). The oligo-nucleotide primers used for RT-PCR were: 5′-TTTCTCGAGTGGGGAGAATGGGGGCCTCTTC-3′ for forward and 5′-TTTGGTACCTTAGTGATGGTGGTGGTG GTG-3′ for reverse.

The protein sample used for western blot was prepared from the culture supernatant. Sufficient amount of cells was obtained by the two-stage culture method developed in our laboratory previously (*14*). Briefly, in the first stage for cell proliferation, cells were grown to confluency in 150-mm dish filled with 25 ml of α-MEM (Gibco/Invitrogen) containing 10% FBS, and then shifted to the next culture stage. For the second culture stage for protein production, cells were cultured in ASF104N medium (Ajinomoto Co., Inc., Tokyo) at 37°C and 5% CO_2 for 36 h. The culture supernatant was concentrated with Amicon Ultra-15 (Millipore, Billerica, MA), and the produced protein was examined by western blot. α-MEM and IMDM media showed equally better cell proliferation rate than other media. Therefore, α-MEM was adopted for the further experiments.

Purification of SCR1-3—MTX-resistant cells were cultured statically up to 70% confluency. Then, the medium was changed to fresh one containing 1 mM sodium butyrate and cells were grown for another 24 h to avoid gene silencing of the inserted pCXD-SCR1-3 (*18*). After washing by PBS, cells were further grown in ASF104N medium (Ajinomoto) containing 100 nM MTX (see details in 'Results' section) and antibiotics for 3 days. Culture supernatant was collected, centrifuged at 1,000*g* for 4 min, and filtrated through 0.22 μm filter to separate protein from cell debris. The solution containing the protein of interest was applied to the column of immobilized metal-ion-affinity chromatography using Histrap HP (GE Healthcare UK, Buckinghamshire, UK), and eluted with 20 mM phosphate buffer containing 125 mM, 250 mM and 500 mM imidazole stepwisely. The collected fractions, which were detectable by CBB staining with sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE), were gathered and dialyzed by changing the buffer to PBS for 24 h at 4°C. The membrane used for dialysis was Spectra/Por 1.1 Biotech Membrane MWCO8000 (Spectrum Laboratories, Inc., Rancho Dominguez, CA), and the dialysed protein solution was ultrafiltrated by Amicon Ultra-15 (Millipore). Finally, concentration of protein was measured with BCA protein assay kit [Thermo Fisher Scientific Inc. (Pierce), Rockford, IL].

SDS–PAGE and Western Blot—SDS–PAGE was performed in 12% gels under reduced condition and commercially available protein molecular weight standards (Bio-Rad, Tokyo) were used for reference. Gels were stained with Bio-safe CBB G-250 (Bio-Rad) and silver (Daiichi Fine Chemical Co., Ltd., Toyama), For western blotting, proteins were transferred electrophoretically onto a nitrocellulose membrane, and the membrane was incubated for cross-reaction for 1 h with the mouse anti-His (C-term) monoclonal antibody (Invitrogen) in diluted 5,000-fold in PBS containing 0.2% Tween-20 and 3% skim milk. The secondary antibody conjugated to horseradish peroxidase of ECL plus Western Blotting Detection Reagents (GE Healthcare UK) was used for detection in diluted 10,000-fold. Detection by chemiluminescence was performed by means of LAS1000 (Fuji Film, Tokyo).

Haemolytic Assay—Normal human serum (NHS) measuring 25 μl in diluted 4-fold was added to 25 μl of incremental concentrations of SCR1-3 diluted by PBS in 96-well plate. Then, 25 μl of sheep whole-blood alsever sterile as a source of sheep red blood cells (SRBC) in diluted 10-fold was added and incubated at 37°C for 30 min. Samples were centrifuged at 700*g* and 50 μl of the supernatant were collected. Lysis of the red cells was assessed spectrophotometrically at 415 nm. Inhibition of complement activity by SCR1-3 was read as a percent of lysis (haemolytic index).

Assay of the Suppression Effect of SCR1-3 on the Necrosis of PA-6 Cells—Complement control activity of SCR1-3 was measured by observing the necrosis of stromal cell line (PA-6) originated from a mouse skull that was cultured with α-MEM medium containing 10% FBS. Cells were washed with PBS twice after reaching 100% confluency, and then 250 μl of α-MEM together with NHS and 185 nM, 740 nM and 1850 nM SCR1-3 were added to the cells, respectively. Cells were incubated at 37°C for 1.5 h. Inactivated NHS that was heated at 56°C for 1 h was used to add to PA-6 for comparison. Cell necrosis and morphology change were observed with the microscope (Carl Zeiss, Germany).

Cleavage of Sugar Chain of SCR1-3 and Comparison of Activity—Sugar chain of SCR1-3 was cleaved by glycosidase of N-linked oligosaccharide, PNGase F (Sigma). Ten units of PNGase F was added to 200 μg of SCR1-3, and then incubated at 37°C for 48 h. The cleavage of sugar chain was confirmed by SDS–PAGE and CBB staining.

The difference of activity between SCR1-3 with and without sugar chain was investigated by checking the viability of CHO DG44 in the presence of NHS. CHO DG44 cells were seeded at 10,000 cells/well in 96 well plate and cultured in α-MEM/10% FBS for 2 days. Then, medium was removed and washed by PBS. After that, cells were cultured in100 μl of NHS in diluted 2-fold and 100 μl of 3 mg/ml anti-CHO antibody

(Cygnus Technologies, Inc.), 10 μl of SCR1-3 (final 10 μM) with and without sugar chain were added, respectively. The plate was incubated in CO_2 incubator for 16 h. Each well was washed with PBS and trypsinized for 5 min. The number of cells was counted by Burker-Turk hemocytometer and cell viability was calculated.

RESULTS

The scheme for construction of SCR1-3 expression plasmid is shown in Fig. 1A. Total RNA was extracted from the CHO35.6 cell from which sCR1 was secreted and thus concerned plasmid vector contained SCR1-3, a main functional domain of sCR1. After reverse transcription, cDNA was refined, and then amplified by PCR. The obtained fragment was introduced into pBluescript and followed by its cloning. The DNA sequence was confirmed, and then the purified DNA was inserted in pCX-N2L, an expression vector for animal cells, to form pCX-N2L-SCR1-3. In order to improve amplification of SCR1-3 gene, dhfr segment, a gene containing SV40 promoter and poly(A) signal which were useful to gene amplification by induction with MTX, was cut out from pSV2-dhfr, inserted into pBluescript and followed by cloning. The fragment encoding dhfr was digested by *Sal*I and *Spe*I, and introduced into pCX-N2L-SCR1-3 to form pCXD-SCR1-3. The newly formed plasmid was digested by restriction enzyme *Bgl*II, and the digested bands were investigated by agarose gel electrophoresis to confirm whether the dhfr gene had been inserted. Two bands of 6 kb and 3 kb were found after the digestion indicating that dhfr gene had been inserted correctly (Fig. 1B). The confirmed plasmid vector was then transfected into CHO DG44 cell by lipofection method. Because CHO DG44 cell, a DHFR-deficient cell, can only grow in medium comprising nucleic acids, it was cultured in α-MEM medium containing nucleic acid firstly. After transfection, the culture medium was exchanged to nucleic acid free, in which only the cells that maintained the plasmid containing dhfr gene were viable, and positive clones were screened. The culture for selection was continued for 2 weeks to obtain stable expressing cells. The selected clones were subject to RT-PCR to confirm the existence of SCR1-3 gene (Fig. 1C).

For mass production of protein, a two-stage culture method developed by us was employed by which cell proliferation and protein production were performed separately. Cells were cultured in serum medium to accelerate cell proliferation firstly, and then cultured in serum free medium to reduce the influence of proteinaceous substances from the serum in the next step of column chromatography. The secreted SCR1-3 in medium was confirmed by western blotting after the second-stage culture for protein production, and the targeted protein was probed by anti-His-tag antibody. In this case, the culture supernatant was concentrated by ultra-filtration because it was presumed that the protein concentration was low. The result showed that SCR1-3 was secreted by cells in the medium (Fig. 2A). In order to enhance the expression of SCR1-3, the selected positive clones were further cultivated in the medium added with MTX. The concentration of MTX was increased from 50 to 350 nM, and from 350 to 1,000 nM, stepwisely, and MTX-resistant cells were screened at each stage. The expressed protein was examined by western blot after the second stage for protein production. Although the expression of SCR1-3 increased greatly with the treatment of MTX, no significant differences in the amount of SCR1-3 expression were found among cells treated with 50, 350 and 1,000 nM of MTX (Fig. 2B). On the other hand, the cell proliferation rate during the 3-day culture was found decreasing significantly in the presence of 1,000 nM MTX, whereas 100 nM appeared not to influence cell proliferation as compared with those cultured in the absence of MTX (Fig. 2C). Therefore, 100 nM MTX was used for the further experiments.

In order to suppress the silencing expression of target protein caused by a virus originated promoter in the constructed plasmid, sodium butyrate, an inhibitor of histone deacetylase which is known to inactivate the gene silencing, was added before the stage of protein production culture. In order to determine the optimum concentration of sodium butyrate for cell culture, the expression amount was confirmed by western blotting using 24-h culture added with sodium butyrate at the different concentrations of 0.5 mM, 1 mM and 2 mM, respectively. High amount of SCR1-3 expression was confirmed at the concentration of 1 mM or more (Fig. 2D, lanes 4 and 5). However, microscopic observation showed that number of dead cells increased in the presence of 2 mM sodium butyrate (Fig. 2E). Therefore, 1 mM butyric sodium was used hereafter in the study.

The culture supernatant containing SCR1-3 was applied on Histrap HP (GE Healthcare UK), a metal-chelate-affinity chromatography. The column was packed with Ni Sepharose resin, which had high binding capacity that ensured reliable capture of His-tagged proteins in purifications. Protein elution from the column was performed with the buffer containing imidazole at 125 mM, 250 mM and 500 mM, stepwisely. SCR1-3 was detected in the fractions eluted with 125 mM imidazole. It was shown that only SCR1-3 appeared as a single band on the SDS–PAGE gel stained with CBB dye (Fig. 3A). The SCR1-3 solution was dialyzed by using PBS as a dialyzing buffer to remove imidazole, then concentrated by ultra filtration using AmiconUltra (Millipore). BCA protein assay showed that the final yielded amount of SCR1-3 from the constructed expression system with sodium butyrate was 1 mg/1l in the culture supernatant. In contrast, yield of existing method without sodium butyrate was 0.5 mg/1l.

To examine the suppression activity of the purified SCR1-3 on complement-mediated inflammation, assays using sheep erythrocytes (Fig. 3B) and PA6 cell (Fig. 3C), the latter is the bone marrow stromal cell from mouse skull, were performed in the presence of NHS because human complement system is known to induce haemolysis on sheep erythrocytes and necrosis on PA-6 cells.

The results showed SCR1-3 suppressed haemolysis depending on its concentration when using NHS as the complement source and sheep erythrocytes as the red blood source (Fig. 3B). Furthermore, microscopic observation indicated distinct morphological difference between the PA6 cells treated with only NHS and those

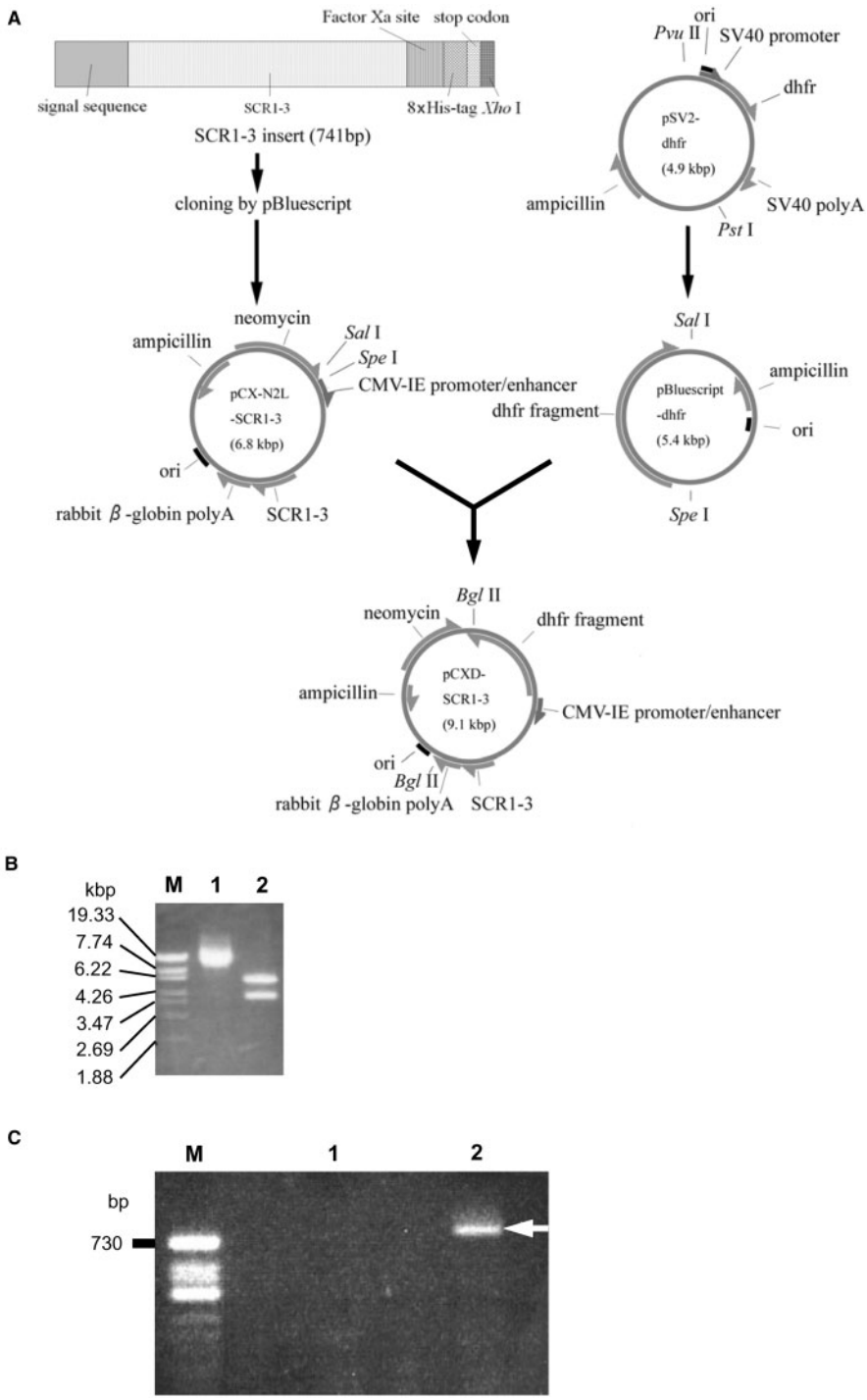

Fig. 1. **(A) A schematic diagram of pCXD-SCR1-3 and its construction scheme.** The SV40 promoter and the dhfr genes were cut out from pSV2-dhfr by restriction enzymes, *Pvu*II and *Pst*I. The fragment containing SV40 promoter and SV40 poly(A) signal was inserted into pBluescript, which was named as pBluescript-dhfr. SCR1-3 gene containing sequences of Factor Xa, 8xHis-tag, stop codon and *Xho*I site at the 3′ end was amplified by PCR, and the PCR product was inserted into pBluescript. The fragment encoding SCR1-3 in pBluescript was further digested by *Sma*I and *Hinc*II, and ligated into pCX-N2L comprising CMV-IE promoter/enhancer and rabbit β-globin poly(A) signal. The formed pCX-N2L-SCR1-3 and vector of pBluescript-dhfr were digested by *Sal*I and *Spe*I, respectively, and the fragment encoding dhfr from pBluescript-dhfr was ligated into pCX-N2L-SCR1-3. This constructed plasmid was named as pCXD-SCR1-3. (B) Analysis of pCXD-SCR1-3 by restriction enzyme. Two *Bgl*II sites were derived from pCX-N2L and inserted dhfr fragment, respectively. Therefore, if dhfr fragment was inserted correctly, *Bgl*II cleaves the plasmid at two sites and generates 3 kb and 6 kb fragments. Lane 1, no digestion; lane 2, digested by *Bgl*II; lane M, molecular weight marker. Concentration of agarose in gel was 1.5%. (C) RT-PCR analysis of CHO DG44 cells. Lane 1, CHO DG44 cells without transfection; lane 2, CHO DG44 cells with transfection. It is expected that the band size of SCR1-3 is ~770 bp (indicated by the arrow). Lane M is molecular weight marker. Three percent agarose gel was used.

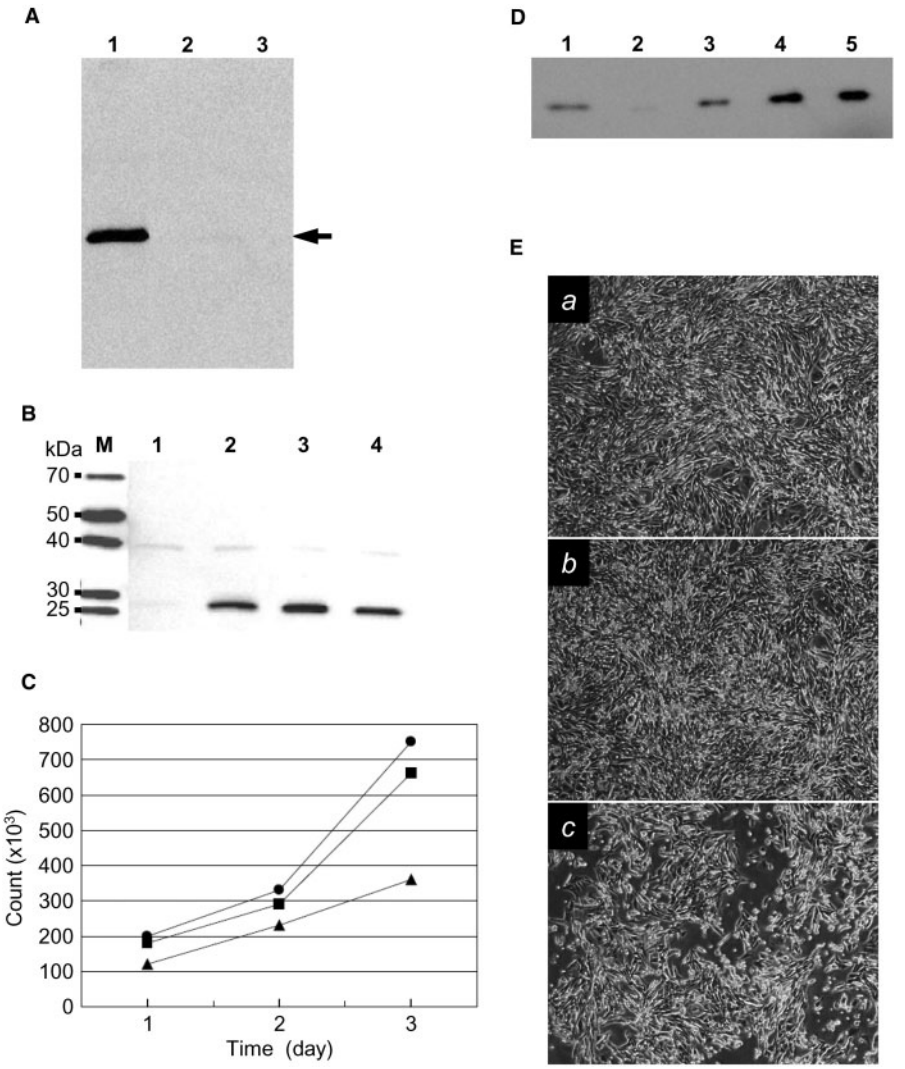

Fig. 2. **(A) Western blotting analysis of the concentrated supernatant of cell culture.** Lane 1, the concentrated culture supernatant in ASF104N medium; lane 2, homogenate of transfected DG44 cell; lane 3, the concentrated supernatant of non-transfected CHO DG44 cell culture in α-MEM/10%FBS medium. Twenty micro litres of sample each was subjected to SDS–PAGE under reducing condition. SCR1-3 is indicated by the arrow. (B) Western blotting analysis of the supernatant of MTX-resistant cell culture in α-MEM/10% FBS medium containing MTX. The concentration of MTX: Lane 1, 0 nM (no MTX-resistance); lane 2, 50 nM; lane 3, 350 nM; lane 4, 1,000 nM. Twenty micro litres of sample solution each was subjected to SDS–PAGE under reducing condition. Lane M indicates molecular weight marker. (C) Time course of cell proliferation at different concentrations of MTX. Transfected DG44 cells were seeded at 1×10^5 cells and cultured for 3 days in α-MEM/10%FBS medium containing MTX at the various concentrations. The concentration of MTX was 1,000 nM (filled triangle), 100 nM (filled square), 0 nM (filled circle). Meanwhile, the number of cells was counted by Burker-Turk hematocytometer. (D) The optimization of sodium butylate (NaBu) concentration in α-MEM/10%FBS medium by western blotting. Cells were cultured in α-MEM/10%FBS containing NaBu at the various concentrations for 24 h. Supernatants of cell culture were collected and subjected to SDS–PAGE under reducing condition. Lanes 1 and 2, without NaBu; lane 3, 0.5 mM; lane 4, 1 mM; lane 5, 2 mM NaBu. Samples for lane 2 through 5 were used in diluted 2-fold by PBS(–). (E) The micrographs of CHO DG44 cells treated by NaBu. The concentration of NaBu was 0.5 mM (a), 1 mM (b) and 2 mM (c), respectively.

treated with NHS in the presence of SCR1-3. It was obvious that the number of the destructed PA6 cells was decreased as the concentration of SCR1-3 increased (Fig. 3C). Both experimental results imply that SCR1-3 could suppress complement attack and protect cells from damage depending on its concentration.

In order to confirm whether the purified SCR1-3 possessed N-linked sugar chain, SCR1-3 was treated with PNGase F, which is a glycosidase to dissociate N-linked sugar chains from polypeptides. SDS–PAGE showed a decreased molecular mass of SCR1-3 after PNGase F treatment (Fig. 4A) and sugar chain of SCR1-3 did not influence the complement control activity greatly because no significant difference could be distinguished between SCR1-3 with and without sugar chain (Fig. 4B).

DISCUSSION

The mammalian expression system of SCR1-3 was constructed with CHO DG44 cell. The plasmid for

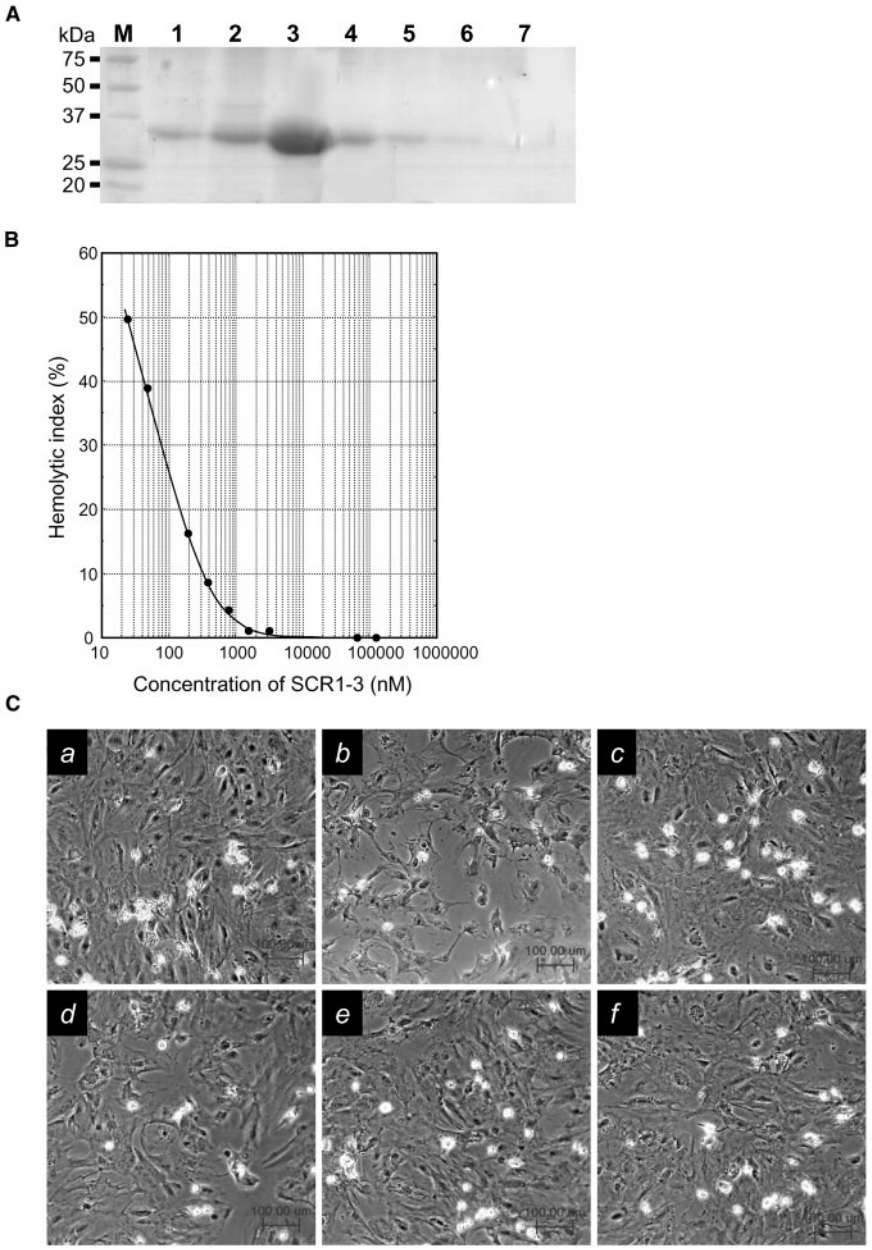

Fig. 3. **(A) CBB-stained SDS–PAGE of fraction of SCR1-3 purification by Histrap column.** One litre of culture supernatant in ASF104N was injected to Histrap column. Affinity column chromatography was performed with peristaltic pump at flow rate of 1 ml/min at 4°C. Line 1, flow-through fraction; lane 2, eluent with wash buffer; lanes 3 and 4, eluent fractions with 125 mM imidazol; lanes 5 and 6, eluent fractions with 250 mM imidazol; lane 7, eluent with 500 mM imidazol. Lane M, molecular weight marker. (B) Haemolytic assay on SRBC added with purified SCR1-3. The effect of SCR1-3 on inhibition of complement-mediated SRBC lysis at various concentration of SCR1-3 in NHS in diluted 4-fold was examined. Sheep whole-blood alsever sterile as a source of SRBC diluted 10-fold was added to SCR1-3 in diluted by PBS in 96 well plate and lysis of the red cells was assessed spectrophotometrically at 415 nm. Inhibition of complement activity by SCR1-3 was read as a percent of lysis. (C) Assay of the suppression effect of SCR1-3 on the necrosis of PA-6 cells. Suppression effect of SCR1-3 was observed by using the stromal cell line (PA-6) originated from mouse skull that was cultured with α-MEM medium containing 10%FBS (*a*), with treatment by NHS (*b*), with treatment by inactivated NHS (*c*), with treatment by NHS in the presence of 185 nM SCR1-3 (*d*), with treatment by NHS in the presence of 740 nM SCR1-3 (*e*), with treatment by NHS in the presence of 1850 nM SCR1-3 (*f*).

expression consists of SCR1-3 coding region and dhfr gene, the latter is a MTX-dependent gene which can enhance amplification by the addition of MTX (*16*). The insertion of dhfr gene was confirmed by electrophoresis after the digestion with restrict enzyme *Bgl*II because two fragments were observed as expected (Fig. 1B). This fact indicated that dhfr gene was inserted into the constructed plasmid correctly. Meanwhile, SCR1-3 was also confirmed by DNA sequencing (data not shown). Transcription of SCR1-3 in the transfected CHO DG44

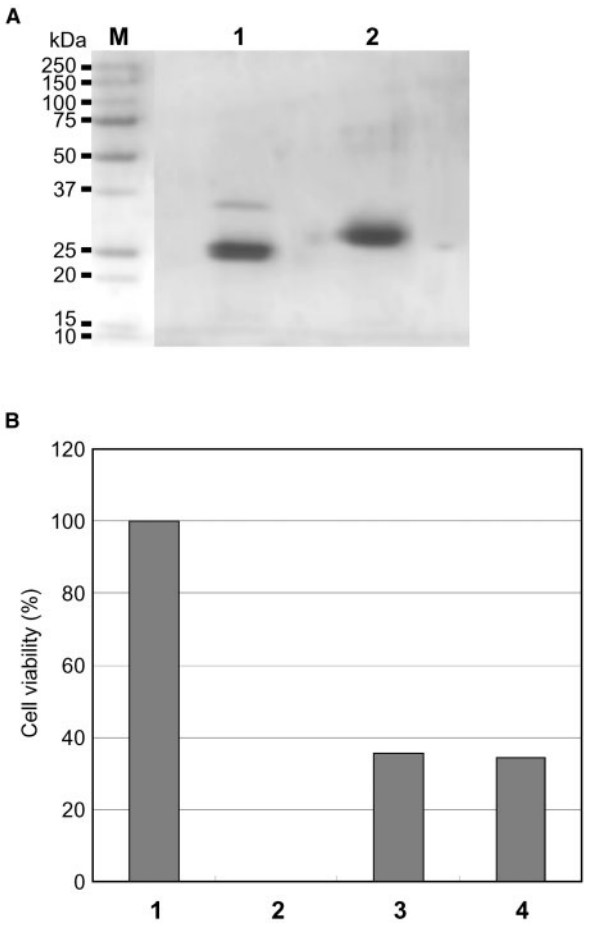

Fig. 4. **(A) CBB-stained SDS–PAGE of deglycosylated and intact SCR1-3 in reducing condition by 2-ME.** Lane 1, deglycosilated SCR1-3 (~25 kDa); lane 2, SCR1-3 (~27 kDa); lane M, molecular weight marker. The thin band seen above the dominant one in lane 1 corresponds to PNGase F (36 kDa). (B) The difference in suppression activity between SCR1-3 with and that without sugar chain. Cell viability (survived cell number) was scored and shown relatively, as the number of non-treated cells was assumed to be 100%. 1, non-treated cells (100%); 2, cells treated by NHS in the absence of SCR1-3 (0%); 3, cells treated by NHS in the presence of glycosylated SCR1-3 (35.8%); 4, cells treated by NHS in the presence of deglycosylated SCR1-3 (34.3%).

cell was confirmed by RT-PCR, and the result showed that the expression vector was correctly constructed (Fig. 1C).

The expression of SCR1-3 by CHO DG44 cell was also confirmed by collecting SCR1-3 secreted in the supernatant of cell-cultured medium and detected by western blotting. Although many efforts have been done to optimize cell proliferation conditions so as to achieve high performance in the purification of the desired protein, the final yield of SCR1-3 from the constructed system appears smaller than that of the over-expression system using *E. coli* as a host cell (*19*). This is acceptable because we used CHO DG44 cell as a host cell to investigate and characterize glycosylated SCR1-3 but the *E. coli* system can hardly carry out such kind of assays. The merit of our expression and purification system is that the protein folds spontaneously with glycosylation and is secreted across cell membranes to the culture medium, and therefore no additional artificial treatments are required.

In order to improve high yield of SCR1-3, a method using MTX-dependent gene amplification was adopted in our study because this gene is usually used for increasing the protein production in mammalian cell. As expected, the result showed that the production of SCR1-3 from the MTX-resistant cells was increased. However, as shown in Fig. 2B and C, MTX at a high concentration did not show significant effect. To the contrary, high concentration of MTX suppressed the cell growth because MTX inhibited *de novo* synthesis of nucleic acids. Another possible reason for the low yield might be due to gene silencing. Many transgenes under the control of viral promoters have been found to be silenced. It was reported that sodium butyrate is one of the regents that can reactivate the silenced gene (*18*) when supplied at an appropriate concentration. Our results showed that sodium butyrate at both 1 mM and 2 mM concentrations did improve the production of SCR1-3, but 1 mM sodium butyrate appeared more effective on cell proliferation in comparison with that at 2 mM (Fig. 2D and E). Moreover, the final yield of SCR1-3 from the constructed expression system with sodium butyrate was 1 mg/1l, which is twice of that of existing method without sodium butyrate. This result suggests that our method combining MTX and butyrate improves the production of SCR1-3.

It has been known that complement components C1–C9 exist in human serum, and their depositions to the surface of target cells causes terminate decompositions when the target cells are recognized. It was reported that membrane attack complex (MAC) consisting of C5b–C9 complex is formed eventually which makes holes on the cell membrane, and causes cell necrosis (*20*). SCR1-3 is a domain of CR1 for binding to complement component C4b (*21*, *22*), and it was reported that SCR1-3 itself has the complement control activity even though its effect is not so high (*23*, *24*). We examined whether SCR1-3 purified in the study possesses the suppression activity on cell damage after the addition of human serum to cells such as SRBC and mouse's skull stromal cell (PA-6). The results showed that SRBC was haemolysed (Fig. 3B) and PA-6 necrosis occurred in the presence of human serum (Fig. 3C *b*).

It appeared reasonable to ascribe that the cell damage, which caused after the addition of a human serum, was due to the complement reaction since the cell damage was not observed when human serum containing inactivated complement components was applied onto PA-6 cells (Fig. 3C *c*). The suppression effect of SCR1-3 on the necrosis of PA-6 cells appeared lower than that of total sCR1 reported by us previously (*14*). However, it is consistent with the other report, which shows that the activity of SCR1-3 is lower than that of total sCR1 in haemolysis (*24*). On the other hand, cell damage could be cured by the addition of SCR1-3 (Fig. 3B *d–f*), and the increasing concentration of SCR1-3 resulted in better cell recovery from damage (Fig. 3B *e and f*). These facts

indicated that SCR1-3 possesses complement control activity to suppress complement attack.

As mentioned above, we confirmed that the purified SCR1-3 could regulate complement activity depending on its concentration in both haemolytic assay and necrosis assay. Both experimental results imply that SCR1-3 could suppress complement attack and protect cells from damage.

In order to investigate whether sugar chains of SCR1-3 are necessary for the inhibition activity on the complement-mediated inflammation, the sugar chains were cut out with an endoglycosidase. Peptide-N4-(acetyl-β-glucosaminyl)-asparagine amidase (PNGase F, EC 3.5.1.52) is known to be the most effective enzyme which can remove N-linked type oligosaccharide from glycoproteins almost completely. Although endoglycosidases of Endoglycosidase H series and F series are often used for the cleavage of sugar chains, they are not suitable for deglycosilation of general N-linked sugar chains due to the existence of specificity which leaves with one *N*-acetylglucosamine residue binding to asparagin residue of protein (*25*). As compared with intact SCR1-3 of 27–28 kDa prepared from our system, SCR1-3 treated with PNGase F lost 1–2 kDa in molecular mass. The deglycosilated SCR1-3 was assumed to be about 23–25 kDa. Figure 4A showed that the sugar chain of SCR1-3 was cut by the deglycosilation with PNGase F because the molecular mass of SCR1-3 (with His-tag) predicted from the amino acid sequence was 22,876 Da (~22.9 kDa), which showed good agreement with the above figure. Furthermore, it was also confirmed that the sugar chain was N-linkage type because PNGase F is an enzyme capable of removing N-linked type sugar chain.

We constructed the expression system using CHO DG44 cell as a host because CHO DG44 cell can secrete glycosylated protein. It has been reported that glycosylation of CR1 influenced the proper expression of its function (*15*, *26*). From the above results, the candidate sites of N-linked complex oligosaccharides are confirmed existing in SCR1-3; thus, we further investigated the participation of the sugar chain of SCR1-3 in the complement control activity. The result indicated that the glycosyrated SCR1-3 expressed in CHO DG44 cells showed almost the same level of complement activity as that without sugar chain (Fig. 4B, lanes 3 and 4).

Yet remained the question whether the species and linkage pattern of N-linked oligosaccharides were the same between the SCR1-3 expressed in CHO DG44 cell and that *in vivo* (human). The significance of the function of sugar chains resided in SCR1-3 should be reconsidered and investigated in further detail.

In conclusion, we successfully constructed an expression system of SCR1-3 in CHO DG44 cell and recovered a lot of protein. The purified SCR1-3 was confirmed to possess the inhibition activity to regulate complement-mediated inflammation, and SCR1-3 in our CHO expression system contained N-linked oligosaccharide although the sugar chain of SCR1-3 appeared rarely important for inhibition activity to suppress complement-mediated inflammation. Further investigation in this regard still remains.

FUNDING

Grants-in-aid for scientific research (B) from Ministry of Education, Culture, Sports, Science and Technology of Japan (to NI).

CONFLICT OF INTEREST

None declared.

REFERENCES

1. Fearon, D.T. (1979) Regulation of the amplification C3 convertase of human complement by an inhibitory protein isolated from human erythrocyte membrane. *Proc. Natl Acad. Sci. USA* **76**, 5867–5871
2. Fearon, D.T. (1980) Identification of the membrane glycoprotein that is the C3b receptor of the human erythrocyte, polymorphonuclear leukocyte, B lymphocyte, and monocyte. *J. Exp. Med.* **152**, 20–30
3. Krych, M. and Atkinson, J.P. (2001) Structure-function relationships of complement receptor type 1. *Immunol. Rev.* **180**, 112–122
4. Ross, G.D., Yount, W.J., Walport, M.J., Winfield, J.B., Parker, C.J., Fuller, C.R., Taylor, R.P., Myones, B.L., and Lachmann, P.J. (1985) Disease-associated loss of erythrocyte complement receptors (CR1, C3b receptors) in patients with systemic lupus erythematosus and other diseases involving autoantibodies and/or complement activation. *J. Immunol.* **135**, 2005–2014
5. Weisman, H.F., Bartow, T., Leppo, M.K., Marsh, H.C., Carson, G.R., Concino, M.F., Boyle, M.P., Roux, R.H., Weisfeldt, M.L., and Fearon, D.T. (1990) Soluble human complement receptor type 1: in vivo inhibitor of complement suppressing post-ischemic myocardial inflammation and necrosis. *Science* **249**, 146–151
6. Klickstein, L.B., Wong, W.W., Smith, J.A., Weis, J.H., Wilson, J.G., and Fearon, D.T. (1987) Human C3b/C4b receptor (CR1). Demonstration of long homologous repeating domains that are composed of the short consensus repeats characteristics of C3/C4 binding proteins. *J. Exp. Med.* **165**, 1095–1112
7. Krych, M., Hauhart, R., Subramanian, V.B., Yurcisin, B.M., Crimmins, D.L., Hourcade, D.E., and Atkinson, J.P. (1999) Decay accelerating activity of complement receptor type 1 (CD35). Two active sites are required for dissociating C5 convertases. *J. Biol. Chem.* **274**, 31160–31168
8. Krych, M., Hauhart, R., Porzukowiak, T., and Atkinson, J.P. (2005) Synergy between two active sites of human complement receptor type 1 (CD35) in complement regulation: implications for the structure of the classical pathway C3 convertase and generation of more potent inhibitors. *J. Immunol.* **175**, 4528–4535
9. Janssen, B.J., Christodoulidou, A., McCarthy, A., Lambris, J.D., and Gros, P. (2006) Structure of C3b reveals conformational changes that underlie complement activity. *Nature* **444**, 213–216
10. Ajees, A.A., Gunasekaran, K., Volanakis, J.E., Narayana, S.V., Kotwal, G.J., and Murthy, H.M. (2006) The structure of complement C3b provides insights into complement activation and regulation. *Nature* **444**, 221–225
11. Krishnan, V., Xu, Y., Macon, K., Volanakis, J.E., and Narayana, S.V. (2007) The crystal structure of C2a, the catalytic fragment of classical pathway C3 and C5 convertase of human complement. *J. Mol. Biol.* **367**, 224–233
12. Furtado, P.B., Huang, C.Y., Ihyembe, D., Hammond, R.A., Marsh, H.C., and Perkins, S.J. (2008) The partly folded back solution structure arrangement of the 30 SCR domains in human complement receptor type 1 (CR1) permits access to its C3b and C4b ligands. *J. Mol. Biol.* **375**, 102–118

13. Smith, B.O., Mallin, R.L., Krych, M., Wang, X., Hauhart, R.E., Bromek, K., Uhrin, D., Atkinson, J.P., and Barlow, P.N. (2002) Structure of the C3b binding site of CR1 (CD35), the immune adherence receptor. *Cell* **108**, 769–780
14. Kato, H., Inoue, T., Ishii, N., Murakami, Y., Matsumura, M., Seya, T., and Wang, P-C. (2002) A novel simple method to purify recombinant soluble human complement receptor type 1 (sCR1) from CHO cell culture. *Biotechnol. Bioprocess. Eng.* **7**, 67–75
15. Lublin, D.M., Griffith, R.C., and Atkinson, J.P. (1986) Influence of glycosylation on allelic and cell-specific Mr variation, receptor processing, and ligand binding of the human complement C3b/C4b receptor. *J. Biol. Chem.* **261**, 5736–5744
16. Gu, M.B., Kern, J.A., Todd, P., and Kompala, D.S. (1992) Effect of amplification of dhfr and lac Z genes on growth and beta-galactosidase expression in susupension cultures of recombinant CHO cells. *Cytotechnology* **9**, 237–245
17. Subramani, S., Mulligan, R., and Berg, P. (1981) Expression of the mouse dihydrofolate reductase complementary deoxyribonucleic acid in simian virus 40 vectors. *Mol. Cell. Biol.* **1**, 854–864
18. Choi, K.H., Basma, H., Singh, J., and Cheng, P.W. (2005) Activation of CMV promoter-controlled glycosyltransferase and beta-galactosidase glycogenes by butyrate, tricostatin A, and 5-aza-2'-deoxycytidine. *Glycoconj. J.* **22**, 63–69
19. Dodd, I., Mossakowska, D.E., Camilleri, P., Haran, M., Hensley, P., Lawlor, E.J., McBay, D.L., Pindar, W., and Smith, R.A.G. (1995) Overexpression in *Escherichia coli*, folding, purification, and characterization of the first three short consensus repeat modules of human complement receptor type 1. *Protein Expres. Purif.* **6**, 727–736
20. Gasque, P. (2004) Complement: a unique innate immune sensor for danger signals. *Mol. Immunol.* **41**, 1089–1098
21. Klickstein, L.B., Bartow, T.J., Miletic, V., Rabson, L.D., Smith, J.A., and Fearon, D.T. (1988) Identification of distinct C3b and C4b recognition sites in the human C3b/C4b receptor (CR1, CD35) by deletion mutagenesis. *J. Exp. Med.* **168**, 1699–1717
22. Krych, M., Hourcade, D., and Atkinson, J.P. (1991) Sites within the complement C3b/C4b receptor important for the specificity of ligand binding. *Proc. Natl Acad. Sci. USA* **88**, 4353–4357
23. Reilly, B.D., Makrides, S.C., Ford, P.J., Marsh, H.C., and Mold, C. (1994) Quantitative analysis of C4b dimer binding to distinct sites on the C3b/C4b receptor (CR1). *J. Biol. Chem.* **269**, 7696–7701
24. Mossakowska, D., Dodd, I., Pindar, W., and Smith, R.A. (1999) Structure-activity relationships within the N-terminal short consensus repeats (SCR) of human CR1 (C3b/C4b receptor, CD35): SCR 3 plays a critical role in inhibition of the classical and alternative pathways of complement activation. *Eur. J. Immunol.* **29**, 1955–1965
25. Tarentino, A.L., Gómez, C.M., and Plummer, T.H. Jr. (1985) Deglycosylation of asparagine-linked glycans by peptide: N-glycosidase F. *Biochemistry* **24**, 4665–4671
26. Ishii, N., Kato, H., and Wang, P-C. (2003) Observation by electron microscopy on recombinant soluble human complement receptor type 1 (sCR1) and its derivative, aglyco-sCR1, from CHO cells. *Enzyme Microbiol. Technol.* **33**, 482–487