JOURNAL OF VIROLOGY, June 1995, p. 3889–3892 Vol. 69, No. 6
0022-538X/95/$04.00+0

The Complement Control Protein Homolog of Herpesvirus Saimiri Regulates Serum Complement by Inhibiting C3 Convertase Activity

WILLIAM L. FODOR,[1]* SCOTT A. ROLLINS,[1] STELLA BIANCO-CARON,[1] RUSSELL P. ROTHER,[1] EDWARD R. GUILMETTE,[1] WILLIS V. BURTON,[1] JENS-CHRISTIAN ALBRECHT,[2] BERNHARD FLECKENSTEIN,[2] AND STEPHEN P. SQUINTO[1]

Alexion Pharmaceuticals Inc., New Haven, Connecticut 06511,[1] and Institut fur Klinische und Molekulare Virologie, Friedrich-Alexander Universitat, D-91054 Erlangen, Germany[2]

Received 8 February 1995/Accepted 14 March 1995

The herpesvirus saimiri genome encodes a complement control protein homolog (CCPH). Stable mammalian cell transfectants expressing a recombinant transmembrane form of CCPH (mCCPH) or a 5′FLAG epitope-tagged mCCPH (5′FLAGmCCPH) conferred resistance to complement-mediated cell damage by inhibiting the lytic activity of human serum complement. The function of CCPH was further defined by showing that the mCCPH and the 5′FLAGmCCPH transfectants inhibited C3 convertase activity and effectively reduced cell surface deposition of the activated complement component, C3d.

Herpesvirus saimiri (HVS), a member of the gammaherpesvirus group (22), is a lymphotrophic virus that causes T-cell lymphomas in New World primates other than its natural host, the squirrel monkey (10). The HVS genome contains two homologs of genes that encode complement-regulatory proteins, a protein that is structurally homologous to C3 convertase inhibitors (CCPH; stands for complement control protein homolog), and a terminal complement inhibitor homolog, HVS-15 (HVSCD59) (1–3, 23). The CCPH gene of HVS exhibits sequence and structural homology to a family of complement-regulatory proteins encoded by the regulators of complement activation gene cluster in humans (1) as well as to the vaccinia virus complement-control protein VCP (1, 14, 15, 17). The complement regulatory group of glycoproteins includes CR2 (CD21 [20]), CR1 (CD35 [13]), MCP (CD46 [16]), DAF (CD55 [5, 19]), C4bp (6), and Factor H, all of which are structurally and functionally related (reviewed in reference 9). The structural similarities include short-consensus-repeating motifs (SCRs) of approximately 60 amino acids that contain 4 invariant cysteine residues and 10 to 19 other highly conserved amino acids. Functionally, these glycoproteins are related by their ability to regulate the amplification of activated complement at the C3 conversion step by interacting with C3b and/or C4b complement components (6, 8, 9, 16). The predicted amino acid sequence homology between the SCRs of CCPH and those of the mammalian C3 convertase inhibitors ranges from 28% identity for SCR1 (CCPH versus MCP and DAF) (1) to 52% identity for SCR3 (CCPH versus C4bp) (1). The HVS CCPH gene encodes two related proteins that differ only in the carboxy-terminal region of the molecule and results from differential splicing of the primary transcript (1). The two CCPH variants contain all four SCR domains; however, one form of the protein is predicted to be anchored to the cell membrane through a putative transmembrane domain (mC CPH), whereas the alternatively spliced form encodes a secreted CCPH protein (sCCPH) (1).

To assess expression and to determine whether the CCPH protein exhibits complement-regulatory function, expression constructs containing a mCCPH or a FLAG epitope-tagged mCCPH (5′FLAGmCCPH) (Fig. 1) were cloned into the mammalian expression vector pcDNAI-AMP (Invitrogen, San Diego, Calif.) and stably transfected into murine BALB/3T3 cells with the drug resistance plasmid SV2Neo (4). The amino-terminal FLAG epitope-tagged version of the molecule was constructed in order to monitor mCCPH protein expression by Western blot (immunoblot) analysis and to provide a second reagent for detection of the recombinantly expressed molecule, i.e., the FLAG-M2 monoclonal antibody (MAb).

Several stable neomycin-resistant clones were assayed for mCCPH or 5′FLAGmCCPH expression by Northern (RNA) blot analysis (4, 7) (data not shown), indirect immunofluorescence, and Western blot analysis. Indirect immunofluorescence of mCCPH-G or 5′FLAGmCCPH-15 transfectants, using a polyclonal antibody raised against CCPH (ALP26; Cocalico Biologicals, Inc., Reamstown, Pa.), revealed cell surface expression for the FLAG epitope-tagged and the wild-type mCCPH proteins (Fig. 2). The 5′FLAGmCCPH protein

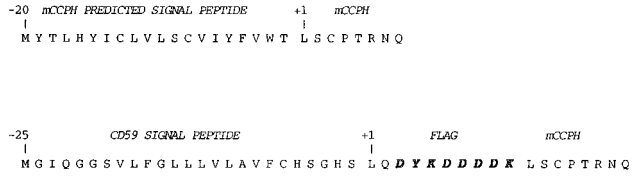

FIG. 1. The amino-terminal amino acid sequences for mCCPH and 5′FLAG-mCCPH, illustrating the leader peptides, the FLAG epitope, and the putative amino-terminal amino acids of the mature protein. The numbers above the sequences indicate the amino acid positions. The 5′FLAGmCCPH was constructed by generating a DNA fragment by PCR with the following primers: the 5′FLAG oligonucleotide 5′-GCCGGCCTGCAG*GACTACAAAGACGATGAC GATAAA*TTAAGCTGTCCTACACGTAACCAG and the 3′ mCCPH oligonucleotide 5′-CTTCCATTTAAAAGATCTTGCGG. The underlined sequences represent unique *Pst*I and *Bgl*II restriction sites that were used for cloning purposes, and the italicized residues represent the FLAG epitope-encoding sequence. The 5′FLAGmCCPH clone was constructed by digesting the mCCPH-pcDNAI-AMP plasmid with *Bam*HI and *Bgl*II to remove the 5′ coding sequences of mCCPH; this was followed by directional ligation of the 5′FLAGmCCPH fragment and the human CD59 leader cDNA fragment (*Bam*HI-*Pst*I) into the mCCPH expression plasmid.

* Corresponding author. Mailing address: Alexion Pharmaceuticals, Department of Molecular Development, 25 Science Park, New Haven, CT 06511. Phone: (203) 776-1790. Fax: (203) 772-3655.

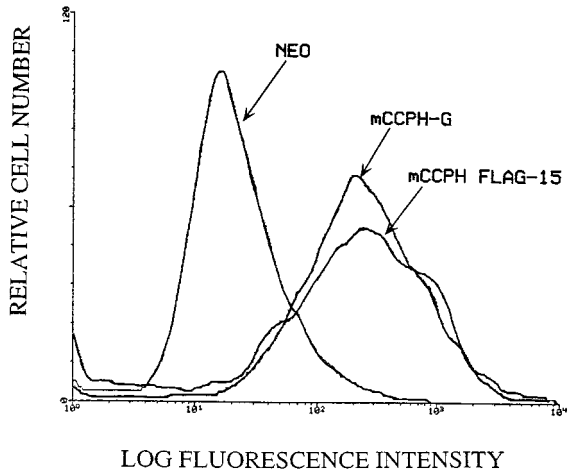

FIG. 2. Indirect immunofluorescence of cell surface-expressed mCCPH. The mCCPH clones, mCCPH-G and 5′FLAGmCCPH-15, and the NEO control cell line were incubated with a rabbit polyclonal antiserum raised against CCPH; this was followed by incubation with a goat anti-rabbit immunoglobulin G fluorescein isothiocyanate-conjugated polyclonal antiserum (final concentration, 15 μg/ml; Zymed). The cells were analyzed by flow cytometry on a Becton-Dickinson FACSort. The labeled curves identify cell surface expression for the different BALB/3T3 mCCPH transfectants compared with that for the neomycin control cells (NEO).

was analyzed further by Western blot analysis, to determine the molecular mass of the protein. Immunoprecipitations of cellular lysates with the MAb SE, specific for CCPH (21), or with the FLAG-M2 antibody (Kodak, New Haven, Conn.) were electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel and transfered to nitrocellulose. The 5′FLAGmCCPH protein was detected with the FLAG-M2 antibody on Western blots and revealed a protein of 39,000 Da, corresponding to the predicted molecular mass of the recombinant protein (Fig. 3).

The primary goal of this study was to determine if mCCPH, expressed in a mammalian cell, could regulate complement. A

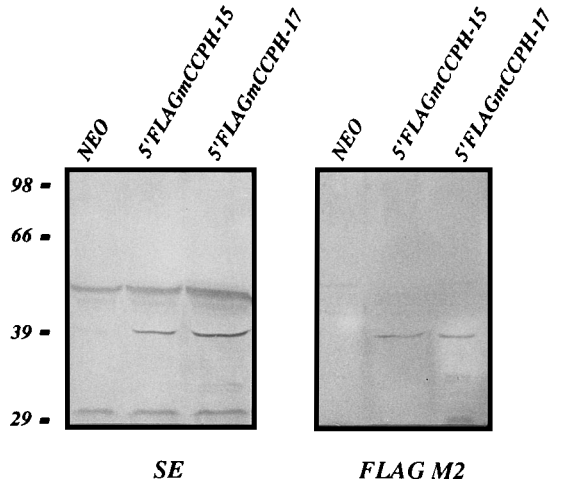

FIG. 3. Immunoprecipitation and Western blot analysis of a NEO control and of 5′FLAGmCCPH-15 and -17 clones. Immunoprecipitations were performed with the anti-CCPH MAb SE or the anti-FLAG MAb M2 (as indicated below the representative blots). The 5′FLAG epitope-tagged mCCPH proteins were then detected with the anti-FLAG M2 antibody. Molecular mass standards (in kilodaltons) are indicated on the left.

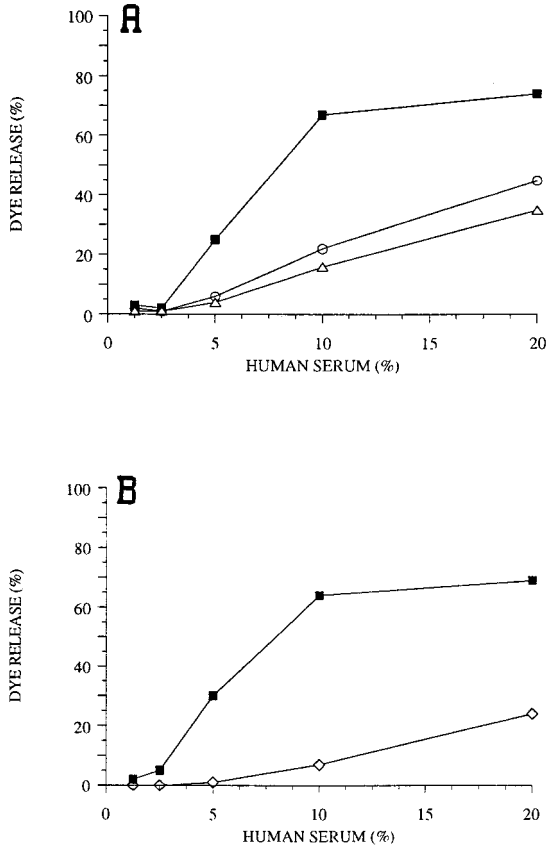

FIG. 4. Complement-mediated dye release assays. Complement-mediated cytolysis was measured by percent dye release (ordinate) with increasing amounts of human whole-serum complement (abscissa). The complement-regulatory activity of stable transfectants mCCPH-G (○) and 5′FLAGmCCPH-15 (△) (A) was compared with that of the human C3 convertase inhibitor DAF (◇) (B). A neomycin (■) control cell line (A and B) was included in each assay and indicates the complement susceptibility of an unprotected cell. Data shown are the means of duplicate determinations from a single experiment, one of three so performed.

complement-mediated dye release assay (23) was used to determine if mCCPH and 5′FLAGmCCPH expressed in BALB/3T3 cells conferred resistance to the lytic effects of serum complement. When human serum was used as a source of complement, the neomycin (NEO) control transfectant exhibited greater than 70% dye release at 20% human serum (Fig. 4), whereas the mCCPH-G and the 5′FLAGmCCPH-15 cell lines exhibited a twofold reduction in dye release (Fig. 4A). The complement-regulating activity of CCPH was more pronounced at 10 and 5% serum concentrations, at which a three- to sixfold difference in percent dye release between the NEO control clone and the mCCPH clones was observed. The control for these experiments was a BALB/3T3 cell line expressing a transfected human DAF cDNA clone. The human DAF cDNA was generated by PCR with oligonucleotide primers homologous to the 5′ and 3′ untranslated regions of DAF (5, 16) and reverse transcribed cDNA from HeLa cells. Transfection of the DAF cDNA into BALB/3T3 cells was performed as described for the mCCPH and 5′FLAGmCCPH expression plasmids. Neomycin-resistant colonies were screened for DAF expression with the DAF MAb BRIC216 (Harlan Bioproducts for Science, Inc., Indianapolis, Ind.). The DAF cell clone illustrated the complement-regulatory activity of a human C3 convertase inhibitor (Fig. 4B). These results show that BALB/3T3

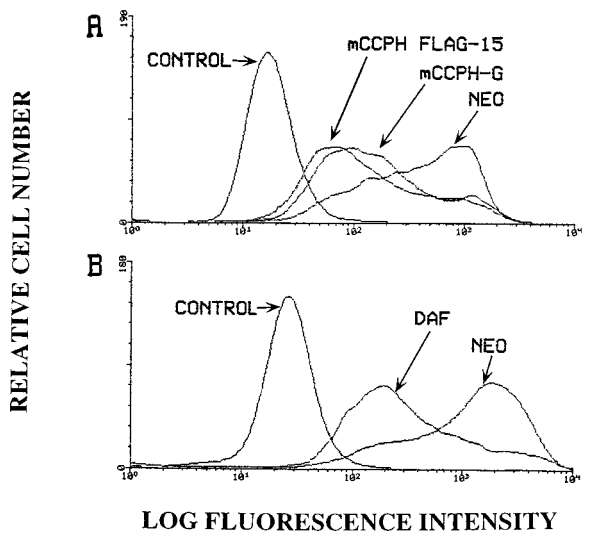

FIG. 5. Complement deposition assays. Indirect immunofluorescence was performed with an anti-C3d MAb on the mCCPH-G and 5′FLAGmCCPH-15 (A) and the DAF (B) positive clones after incubation in 20% C8d human serum. Subsequent to the serum incubations, the cells were incubated in the presence of the anti-C3 MAb C3d (final concentration, 5 μg/ml; Quidel); this was followed by incubation with goat anti-mouse immunoglobulin G fluorescein isothiocyanate-conjugated polyclonal antiserum (final concentration, 15 μg/ml; Zymed). The cells were analyzed by flow cytometry on a Becton-Dickinson FACSort. The labeled curves identify the amount of activated C3 deposited on the cell surface for the different BALB/3T3 transfectants as well as the neomycin control cells (NEO). Nonspecific binding of the anti-C3 antibody was determined in the absence of serum (CONTROL).

transfectants expressing either form of mCCPH confer protection against human serum complement in a dose-dependent manner with respect to increased complement input.

The whole-serum-complement-mediated dye release assays demonstrated that mCCPH functioned to inhibit complement-mediated lysis; however, these assays did not define the mechanism by which mCCPH regulated the complement system. To further define the functional mechanism of mCCPH, complement deposition assays were performed (23). The assay involves depositing early complement components on the cell surface in the absence of cell lysis. The indirect immunofluorescence profiles of cells that were incubated in 20% human C8-deficient serum (C8d) (Quidel, San Diego, Calif.) and then incubated with the MAb anti-C3d (Quidel, San Diego, Calif.), specific for the C3d component of activated human complement, are shown in Fig. 5. The mCCPH-G and 5′FLAGmC CPH-15 transfectants clearly inhibited C3 deposition when compared with the NEO control clone (Fig. 5A). The DAF clone revealed a similar C3 deposition FACS profile at 20% human serum and illustrated the function of a human C3 convertase inhibitor (Fig. 5B). These results establish that mC CPH and an amino-terminal FLAG epitope-tagged form of mCCPH inhibit the classical pathway of complement activation at the C3 conversion step and are functionally related to the regulators of complement activation gene members. This study also demonstrated that amino-terminal manipulation of the protein did not alter cell surface expression and that the molecule retained complement-regulating activity.

Herpesvirus saimiri represents another example of an etiological organism that encodes complement-regulatory molecules: a C3 convertase inhibitor, mCCPH, and a terminal complement inhibitor, HVSCD59 (23). Inhibiting the lytic effects of serum complement would result in increased survival of HVS. The vaccinia virus VCP protein as well as the herpes simplex virus gC-1 and gC-2 proteins plays a role in enhancing viral survival (references 12, 11, and 18, respectively), and a similar role for mCCPH and HVSCD59 seems likely. It is interesting that HVS encodes two complement inhibitors that function at different points in the complement cascade, potentially allowing the virus to evade host complement-mediated virolysis, gain cellular entry, and protect the infected cell. This is the first example of a virus that has acquired two complement-regulatory molecules that regulate complement using functionally different mechanisms.

We thank Eva Setter, Julie Butte, and Amy Chodera for technical assistance and advice. We also thank Carol Newman for supplying the anti-CCPH antibody SE.

This work was supported in part by the Deutsche Forschungsgemeinschaft (Forschergruppe "DNA-Viren des Hamatopoetischen Systems").

REFERENCES

1. **Albrecht, J.-C., and B. Fleckenstein.** 1992. New member of the multigene family of complement control proteins in herpesvirus saimiri. J. Virol. **66:** 3937–3940.
2. **Albrecht, J.-C., J. Nicholas, D. Biller, K. R. Cameron, B. Biesinger, C. Newman, S. Wittman, M. A. Craxton, H. Coleman, B. Fleckenstein, and R. W. Honess.** 1992. Primary structure of the herpesvirus saimiri genome. J. Virol. **66:**5047–5058.
3. **Albrecht, J.-C., J. Nicholas, K. R. Cameron, C. Newman, B. Fleckenstein, and R. W. Honess.** 1992. Herpesvirus saimiri has a gene specifying a homologue of the cellular membrane glycoprotein CD59. Virology **190:**527–530.
4. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1991. Current protocols in molecular biology, vol. 1. John Wiley & Sons. New York.
5. **Caras, I. W., M. A. Davitz, L. Rhee, G. Weddell, D. W. Martin, Jr., and V. Nussenzweig.** 1987. Cloning of decay-accelerating factor suggests novel use of splicing to generate two proteins. Nature (London) **325:**545–549.
6. **Chung, L. P., D. R. Bently, and K. B. M. Reid.** 1985. Molecular cloning and characterization of the cDNA coding for the C4b-binding protein, a regulatory protein of the classical pathway of the human complement system. Biochem. J. **230:**133–141.
7. **Church, G. H., and W. Gilbert.** 1984. Genomic sequencing. Proc. Natl. Acad. Sci. USA **81:**1991–1995.
8. **Coyne, K. E., S. E. Hall, E. S. Thompson, M. A. Arce, T. Kinoshita, T. Fujita, D. J. Anstee, W. Rosse, and D. M. Lublin.** 1992. Mapping of epitopes, glycosylation sites and complement regulatory domains in human decay accelerating factor. J. Immunol. **149:**2906–2913.
9. **Farries, T. C., and J. P. Atkinson.** 1991. Evolution of the complement system. Immunol. Today **12:**295–300.
10. **Fleckenstein, B., and R. C. Desrosiers.** 1982. Herpesvirus saimiri and herpesvirus ateles, p. 253–332. *In* B. Roizman (ed.), The herpesviruses, vol. 1. Plenum Press, New York.
11. **Harris, S. L., I. Frank, A. Yee, G. H. Cohen, R. J. Eisenberg, and H. M. Friedman.** 1990. Glycoprotein C of herpes simplex virus type I prevents complement-mediated cell lysis and virus neutralization. J. Infect. Dis. **162:**331–337.
12. **Isaacs, S. N., G. J. Kotwal, and B. Moss.** 1992. Vaccinia virus complement-control protein prevents antibody-dependent complement-enhanced neutralization of infectivity and contributes to virulence. Proc. Natl. Acad. Sci. USA **89:**628–632.
13. **Klickstein, L. B., W. W. Wong, J. A. Smith, J. H. Weis, J. G. Wilson, and D. T. Fearon.** 1987. Human C3b/C4b receptor (CR1). Demonstration of long homologous repeating domains that are composed of short consensus repeats characteristic of C3/C4 binding proteins. J. Exp. Med. **165:**1095–1112.
14. **Kotwal, G. J., S. N. Isaacs, R. McKenzie, M. M. Frank, and B. Moss.** 1990. Inhibition of the complement cascade by the major secretory protein of vaccinia virus. Science **250:**827–830.
15. **Kotwal, G. J., and B. Moss.** 1988. Vaccinia virus encodes a secretory polypeptide structurally related to complement control proteins. Nature (London) **335:**176–181.
16. **Lublin, D. M., M. K. Liszewski, T. W. Post, M. A. Arce, M. M. LeBeau, M. B. Rebentish, R. S. Lemons, T. Seya, and J. P. Atkinson.** 1988. Molecular cloning and chromosomal localization of human membrane cofactor protein (MCP). Evidence for the inclusion in the multi-gene family of complement-regulatory proteins. J. Exp. Med. **168:**181–194.
17. **McKenzie, R., G. J. Kotwal, B. Moss, C. H. Hammer, and M. M. Frank.** 1992. Regulation of complement activity by vaccinia virus complement-control protein. J. Infect. Dis. **166:**1245–1250.
18. **McNearney, T. A., C. Odell, V. M. Holers, P. G. Spear, and J. P. Atkinson.** 1987. Herpes simplex virus glycoproteins gC-1 and gC-2 bind to the third

component of complement and provide protection against complement-mediated neutralization of viral infectivity. J. Exp. Med. **166:**1525–1535.

19. **Medof, M. E., D. M. Lublin, V. M. Holers, D. J. Ayers, R. R. Getty, J. F. Leykam, J. P. Atkinson, and M. L. Tykocinski.** 1987. Cloning and characterization of cDNAs encoding the complete sequence of decay accelerating factor of human complement. Proc. Natl. Acad. Sci. USA **84:**2007–2011.
20. **Moore, M. D., N. R. Cooper, B. F. Tack, G. R. Nemerow.** 1987. Molecular cloning of the cDNA encoding the Epstein-Barr virus/C3d receptor (complement receptor type 2) of human B lymphocytes. Proc. Natl. Acad. Sci. USA **84:**9194–9198.
21. **Randall, R. E., C. Newman, and R. W. Honess.** 1984. Isolation and characterization of monoclonal antibodies to structural and nonstructural herpesvirus saimiri proteins. J. Virol. **52:**872–883.
22. **Roizman, B.** 1982. The family herpesviridae: general discription, taxonomy and classification, p. 1–23. *In* B. Roizman (ed.), The herpesviruses, vol. 1. Plenum Press, New York.
23. **Rother, R. P., S. A. Rollins, W. L. Fodor, J.-C. Albrecht, E. Setter, B. Fleckenstein, and S. P. Squinto.** 1994. Inhibition of complement-mediated cytolysis by the terminal complement inhibitor of herpesvirus saimiri. J. Virol. **68:**730–737.