Three SIBLINGs (Small Integrin-Binding Ligand, N-linked Glycoproteins) Enhance Factor H’s Cofactor Activity Enabling MCP-like Cellular Evasion of Complement-mediated Attack*

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Previously we have shown that two members of the newly named SIBLING (small integrin-binding ligand, N-linked glycoproteins) family of proteins, bone sialoprotein, and osteopontin, bound first to a cell surface receptor and then to complement Factor H thereby blocking the lytic activity of the alternative pathway of complement. Another member of this family, dentin matrix protein 1, is shown in this paper to be very similar to osteopontin in that it can bind strongly to Factor H (K∞1 nM) and block the lytic activity through either the vitronectin receptor (αvβ3, integrin) or CD44. Binding of Factor H to SIBLING localized to the cells surface was demonstrated by fluorescence-activated cell sorting. Extensive overlapping fragment analyses suggests that both dentin matrix protein 1 and osteopontin interact with cell surface CD44 through their amino termini. Similar fragments of bone sialoprotein, like the intact protein, did not functionally interact with CD44. All three proteins are shown to act in conjunction with Factor I, a serum protease that, when complexed to appropriate cofactors, stops the lytic pathway by digesting the bound C3b in a series of proteolytic steps. These results show that at least three members of this family confer membrane cofactor protein-like activity (MCP or MCP-like) to any cell through their interaction with Factor H, the major humoral protein that controls ACP. The SIBLING proteins strongly bound in a stoichiometric fashion to complement Factor H, the major humoral protein that controls ACP. Furthermore, it was shown that to have this protective effect, the SIBLING must first bind to the vitronectin receptor (for both) or CD44 (for OPN) and then to Factor H for the complex to inhibit the lysis of the cells. Whenever the SIBLING-Factor H complex was allowed to form before binding to the cell’s surface receptor(s) the protective properties were lost. This loss of activity appeared to be due to a masking of cell receptor(s) by the preformed SIBLING-Factor H complexes. This observation suggests that the functional range of the secreted BSP or OPN is likely to be limited to the distance that they can diffuse to a cell surface receptor before being bound and inactivated by the relatively abundant Factor H in the blood and tissue fluids.

Recently we have proposed that a number of proteins whose genes are clustered together on human chromosome 4 (mouse chromosome 5) are a genetically related family termed SIBLINGs,1 for small integrin-binding ligand, N-linked glycoproteins. While direct comparisons of the primary protein sequences of these proteins would not lead to a hypothesis that these proteins are closely related, a systematic look at the: 1) properties of each exon (containing casein kinase II phosphorylation sites or Arg-Gly-Asp (RGD) integrin-binding tripeptide, polyacidic stretches, etc.); 2) the exons involved in splice variants (identical exons) and, 3) the fact that all introns interrupt the coding sequences only between codons, clearly suggests that these clustered genes are related (1). At this time, the gene products within this family include four acidic proteins: bone sialoprotein (BSP) (2), osteopontin (OPN) (3), dentin matrix protein 1 (DMP1) (4), and dentin sialophosphoprotein (5, 6). Matrix extracellular protein (7) (also known as OF45 (8)) is a positively charged protein that appears to be a more distantly related member of the SIBLING family. Bone acidic glycoprotein-75 (9) may be a member due to several of its biochemical and biological properties but it has not yet been cloned and sequenced.

Except for a high affinity for hydroxy apatite among the acidic members and a universal ability to support cell attachment in vitro (through their RGD integrin-binding tripeptides), very little has been described about the possible common shared functions of the SIBLING proteins. Recently we have shown that both BSP and OPN can protect cells from being lysed by the alternative complement pathway (ACP) (10). Both proteins strongly bound in a stoichiometric fashion to complement Factor H, the major humoral protein that controls ACP. Furthermore, it was shown that to have this protective effect, the SIBLING must first bind to the vitronectin receptor (for both) or CD44 (for OPN) and then to Factor H for the complex to inhibit the lysis of the cells. Whenever the SIBLING-Factor H complex was allowed to form before binding to the cell’s surface receptor(s) the protective properties were lost. This loss of activity appeared to be due to a masking of cell receptor-binding sites by the preformed SIBLING-Factor H complexes. This observation suggests that the functional range of the secreted BSP or OPN is likely to be limited to the distance that they can diffuse to a cell surface receptor before being bound and inactivated by the relatively abundant Factor H in the blood and tissue fluids.

DMP1 was first cloned from a rat cDNA library by George et al. (11) and was shown to have an acidic primary structure as well as numerous phosphorylation sites. The integrin-binding tripeptide, RGD, first observed in the rat cDNA sequence and confirmed in many species since, has been shown to support cell attachment by some cells in vitro (12). Although it was first proposed to be dentin specific, the message for DMP1 has been identified in a number of other mineralized tissues as well as brain (6, 13). In 1997, Hirst et al. (14) published the genomic organization of the human DMP1 gene and excluded the locus from a causative role in at least two families with dentinogenesis imperfecta type II (14).

In this paper, we will show that this third SIBLING protein,
DMP1, can also protect tumor cells from attack by the ACP and does so by bridging Factor H to integrins and CD44. Furthermore, the structural similarities and differences between the SIBLING family members are exploited to investigate the mechanism and sequences involved in complement modulation.

EXPERIMENTAL PROCEDURES

Reagents—Rabbit anti-DMP1 peptide-derived antibody, LF-148, was raised against the human sequences (C/HEPSRKIPRKSRISE and C/LKHIIEESRLTVDAYH) conjugated through the Cys to activated horse radish peroxidase (Pierce Chemical Co., Chicago, IL). This antiserum bound to fragments D6 and D8 (see Fig. 5 under “Results”) in direct ELISA suggesting that both peptides successfully raised useful IgG components. Furthermore, this antiserum recognized recombinant mouse DMP1 made in Escherichia coli and full-length bovine DMP1 described below. Normal human serum, purified human complement Factor H protein, and mouse monoclonal antibody against Factor H were obtained from Quidel Corp. (San Diego, CA). A monoclonal antibody against human complement Factor I that blocks Factor I activity (clavage of C3b) (catalog number A247) as well as a monoclonal antibody to Factor I that binds but does not block Factor I function (catalog number A231) were also obtained from Quidel Corp. Polyclonal antibodies against a functional antibody against α1β1 (catalog number MAB1976) were obtained from Chemicon Co. (Temecula, CA). Synthetic purified glycine-arginine-aspartate-serine peptide (GRGDS) was obtained from Calbiochem-NovaBiochem Corp. (La Jolla, CA). Synthetic peptides corresponding to the sequences VKQADSGSSEEKQ (OPN exon 3) and LYNKYDPAVATLWNPSQKGNLLAQ (OPN exon 4) were made and purified by the Peptide Laboratory of the Facility for Biotechnology Resources, Center for Biologies Evaluation and Research, Food and Drug Administration (Bethesda, MD). Preimmune serum, human serum-adsoned goat anti-rabbit IgG conjugated to horseradish peroxidase (1 mg/ml IgG) as well as goat anti-mouse IgG and Recombinant SIBLINGs Enhance Factor H Activity

Production of Recombinant Intact SIBLINGs—A pET-15b vector (Novagen Inc., Madison, WI) which produces polypeptides as fusion products with an amino-terminal polyhistidine sequence followed by a thrombin cleavage site (MGSSHHHHHHSSGLVPRGSH) was used for expression and generation of most of the peptides. Peptides were purified from isopropyl-1-thio-β-D-galactopyranoside-induced log phase E. coli by nickel affinity chromatography (Ni2+-IMAC) following the manufacturer’s protocol. Sequences of primers used to insert the in-frame residues at the carboxyl terminus. The use of pET-22b results in fusion polypeptides with the six His site. The use of pET-22b results in fusion polypeptides with the six His

High Performance Liquid Chromatography—A Shimadzu LC10AS binary gradient system was employed for chromatography separations. Size exclusion chromatography utilized a 1.0 3 30-cm Superose 6 column (Amershams Bioscience, Piscataway, NJ) equilibrated in 0.05 M sodium phosphate, pH 7.4, containing 50% fresh formamidase at a flow rate of 0.5 ml/min. The column was calibrated using commercially available protein standards of known molecular weight (Amersham Bioscience).

Direct ELISA—Greiner high-binding 96-well plates (part number 655061) were coated with 100 µl of high performance liquid chromatography fractions overnight at 4 °C. Plates were washed three times (5 min each) with TBS-Tween and exposed to 100 µl of 1:2000 primary antibody for 1 h at room temperature. Plates were washed three times and exposed to 100 µl of 1:2000 horseradish peroxidase-conjugated goat anti-rabbit IgG. Following a 1-h incubation at room temperature, plates were washed again three times with TBS-Tween and color was developed using 3.3',5,5'-tetramethylbenzidine and H2O2 for 10 min at room temperature. Color development was stopped by the addition of 25 µl of 1 N H2SO4 and analyzed at 450 nm.

Production of Recombinant Intact SIBLINGs—Recombinant human BSP and OPN were made and expressed as described previously (10). For DMP1 expression, an adenoviral construct was generated by subcloning full-length bovine DMP1 cDNA (6, 13) into high expression, replication-deficient adenovirus (Ad5) using the cytomegalovirus promoter. The construct was selected, purified, and expressed following the method described previously for BSF and OPN adeno viruses (10).

Briefly, adenovirus was plaque-selected and propagated on HEK 293 cells (ATCC number CRL1573). Viral particles were purified by twice banding on CsCl and viral titers evaluated by plaque formation of virus dilutions on HEK293 cells (16). Recombinant DMP1 was generated by infecting subconfluent normal human marrow stromal fibroblasts with 10,000 pfu/cell. Harvested serum-free media was subjected to anion exchange chromatography. Native BSP, DMP1, and OPN proteins were purified by dialuting medium from normal human marrow stromal fibroblast cells 1:1 with 40 mM phosphate buffer, pH 7.4, and loading directly on a 5.0 3 2.0-cm column packed with Toyopearl TSK QAE arson. A linear salt gradient from 2.0 to NaCl was employed to separately purify the three proteins to ~95% purity as measured by SDS-PAGE.

Production of Recombinant SIBLING Fragments—A pET-15b vector (Novagen Inc., Madison, WI) which produces polypeptides as fusion products with an amino-terminal polyhistidine sequence followed by a thrombin cleavage site (MGSSHHHHHHSSGLVPRGSH) was used for expression and generation of most of the peptides. Peptides were purified from isopropyl-1-thio-β-D-galactopyranoside-induced log phase E. coli by nickel affinity chromatography (Ni2+-IMAC) following the manufacturer’s protocol. Sequences of primers used to insert the in-frame appropriate restriction sites (NdeI and BamHI) and generate the rele vant SIBLING fragment by PCR for insertion into the vector are given. All OPN fragments were derived from the human sequence (3). The two OPN fragments containing the RGD domain (O4 and O5) were engineered in a pET-22b vector with NcoI sites because of an internal NdeI site. The use of pET-22b results in fusion polypeptides with the six His residues at the carboxyl terminus. The first two DMP1 peptides (D1 and D2) were made using the bovine DMP1 cDNA as template (13) and all others were made using human genomic hDMP1A for the PCR template.

Alternative Complement Mediated Cell Lysis Assay—Murine erythro-leukemia (MEL) cells (a gift of Dr. Marilyn Farquhar, University of California, San Diego, CA) grown in Dulbecco’s modified essential medium containing 10% fetal bovine serum and 4 mM glutamine were rinsed three times with gelatin veronal buffer (GVB, Sigma) containing 2 mM Mg2+ and 8 mM EGTA. Cells were resuspended in GVB-MgEGTA.
**RESULTS**

**DMP1 Exists as a Complex in Serum Bound to Factor H**—We have proposed that, based on a shared chromosomal localization, similarities in exon structure and intron type, that DMP1 belongs to the SIBLING family of proteins (1). We have previously found that two other acidic SIBLING family members, BSP and OPN, are bound to complement Factor H in serum and that disruption of the complex requires heating and reduction (10). The strong (nm) interaction between Factor H and either BSP or OPN (which have no cysteine residues) is non-covalent and required reduction to disrupt due to the unique structure of Factor H. By NMR spectroscopy, BSP and OPN lack ordered structure and exist extended and flexible in solution (1), while Factor H is a large and highly structured protein containing 20 repeated short consensus repeat motifs. The status of DMP1 in human serum was studied by SDS-PAGE and Western blotting as well as by size exclusion chromatography (SEC). When aliquots of normal human serum diluted 1:10 were subjected to SDS-PAGE followed by transfer to nitrocellulose and probing with a peptide-derived antibody against DMP1, immunoreactive bands were readily apparent (Fig. 1A). The migration position of immunoreactive DMP1 shifted upon heating and reduction consistent with the destruction of the DMP1-Factor H complex.

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**FIG. 2. Fluorescence titration.** A, intrinsic tryptophan fluorescence was monitored by excitation at 295 nm and emission from 300 to 500 nm using a Photon Technology International Series M fluorimeter. The initial Factor H concentration was 28 nm and DMP1 was added in nanomolar amounts. Both Factor H and DMP1 were dissolved in Hank’s balanced salt solution. B, the binding curve was determined following calculation of fractional acceptor saturation. Factor H contains 25 tryptophan residues. The molecule’s robust fluorescent emission signal, the contribution of the fluorescent spectra arising from DMP1 was negligible. The fluorescent signal for Factor H was progressively quenched until an equimolar amount of DMP1 was added showing a 1:1 saturable binding. The estimated binding of DMP1 to Factor H is in the nanomolar range.

**FIG. 3. Demonstration of DMP1-factor H binding by FACS analysis.** MEL cells treated as described under “Experimental Procedures” incubated with either PBS or recombinant DMP1 for 10 min at room temperature. The cells were then washed twice and incubated with Alexa Fluor 488-labeled complement protein factor H for 10 min at room temperature. The cells were washed twice, re-suspended in PBS, and then analyzed by FACSCalibur cell sorter equipped with a 488-nm argon laser using Cellquest software. Shaded area under the curve marks the observed profile for the DMP1 + Factor H-treated cells. Only those cells pretreated with the DMP1 bound significant amounts of labeled Factor H.

**FIG. 4. SIBLINGs confer protection from complement-mediated lysis.** MEL cells are lysed by the ACP in human serum and this results in a failure to produce the blue color from MTT (Serum Control). MEL cells in GVB-MgEGTA buffer were treated with 10 μg/ml DMP1, OPN, or BSP prior to the addition of normal human serum diluted 1:10 in GVB-MgEGTA. Following a 2-h incubation, cell viability was monitored by the thiazolyl blue assay. Tissue culture well images were captured using a Polaroid DMC 1e digital microscope camera.

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(gelatin veronal buffer containing 2 mM magnesium and 8 mM EGTA) at a density of 5 × 10⁶ cells/ml. Cells were preincubated with 10 μg of DMP1, BSP, or OPN in 1 ml for 10 min at 37 °C followed by incubation at 37 °C with normal human serum diluted 1:10 in GVB-MgEGTA. After 2 h, cells were harvested for thiazolyl blue viability assay by incubating a 50-μl aliquot of the cell suspension in an equal volume of 1 mg/ml thiazolyl blue (MTT) for 45 min. Cell viability was determined spectrophotometrically by absorbance at 560 nm.

Fluorescence-activated Cell Sorting (FACS) Analysis—MEL cells (2 × 10⁶/ml) were washed twice in PBS and incubated with either PBS or recombinant DMP1 (10 μg/ml) at room temperature. The cells were then washed twice and incubated with 5 μg/ml Alexa Fluor 488 (Molecular Probes, Eugene, OR) -labeled purified human complement protein Factor H (Quidel, San Diego, CA) for 10 min at room temperature. The cells were washed twice, re-suspended in PBS, and then analyzed by FACSCalibur cell sorter equipped with a 488-nm argon laser using Cellquest software (BD PharMingen, Bedford, MA).
SIBLINGs Enhance Factor H Activity

**DMP1 Protects Cells from Alternative Complement Pathway-mediates Lysis**—DMP1 binding to Factor H suggests that this SIBLING may also confer resistance to humoral complement surveillance. The ability of DMP1 to protect cells from complement activity was investigated using the MEL cell line which, when incubated with normal human serum, can be readily assayed for ACP-mediated cell lysis (10, 17). Cell survival was measured by MTT reduction by living mitochondria. Titration with dilutions of normal human serum and time courses were carried out to define optimal incubation conditions. The addition of purified recombinant SIBLING to MEL cells followed by treatment with normal human serum protected the cells from lysis (Fig. 4). The protection of MEL cells from ACP-mediated cell lysis by DMP1 addition exhibited a dose response (data not shown).

**CD44 and α1β3 Are Involved in DMP1 Binding to the Cell Surface**—The SIBLINGs, BSP, and OPN, were previously found to protect cells from complement-mediated lysis through an initial cell surface binding to a receptor. For BSP the membrane receptor was α1β3 while OPN was found to interact with either α1β3 or CD44 (10). The identity of the receptor(s) involved in DMP1 conferred protection from lysis was investigated (Fig. 5). Pretreatment of MEL cells with an anti-α1β3 antibody that blocks ligand binding decreased the protective effect of DMP1, although some protective activity remained. Preincubation with anti-CD44 antibody as well as with hyaluronan, a natural ligand for CD44 (18), also reduced the protective effect of DMP1 addition exhibited a dose response (data not shown).

**DMP1 and Factor H Binding**—Complement Factor H possesses 25 tryptophan residues, while DMP1 contains 2. Thus, the binding between Factor H and DMP1 can be followed by intrinsic tryptophan fluorescence. Titration of purified human complement Factor H with DMP1 was followed by excitation at 295 nm and monitoring emission between 300 and 450 nm. The emission profile of Factor H alone yields a peak at 347 nm (Fig. 2A). The addition of DMP1 in nanomolar increments causes a relative fluorescent intensity quenching. Conversion of the fluorescent intensity titration into a binding curve by determining the fraction of binding sites occupied as the fractional change in fluorescence quenching at 347 nm yields a saturable binding curve (Fig. 2B). By steady state fluorescence, the binding of DMP1 by Factor H is saturable, possesses a 1:1 stoichiometry, and has a binding constant in the nanomolar range. This value is similar to Factor H interactions with BSP and OPN reported earlier.

**DMP1 Bridges Factor H to the Cell Surface**—The direct binding of Factor H to DMP1, while DMP1 is engaged with its cell surface receptor was studied by fluorescence-activated cell sorting. MEL cells incubated with DMP1, briefly washed, incubated with Alexa Fluor 488-conjugated purified human complement protein Factor H, and then analyzed by FACSCalibur cell sorter. The results indicate that a significant association of cell surface Factor H was evident only in the DMP1-treated cells (Fig. 3). Similar shifts were seen when cells were subjected to an initial incubation with BSP and OPN (data not shown).

**DMP1- and OPN-CD44-Binding Domains**—The structural similarities and differences of the SIBLING family members can be exploited to determine the sequences involved in specific binding interactions. A series of overlapping peptides for OPN and DMP1 were created to use in identifying binding sequences (Fig. 6). An in vitro assay for functional binding was designed...
to test each fragment's ability to block intact SIBLING-conferred protection from lysis. First, the cell's αvβ3 integrins were blocked by saturation with GRGDS peptide so that any added OPN or DMP1 should interact only with CD44. Incubation of these αvβ3-blocked cells with DMP1 or OPN fragments was followed, first by treatment with intact DMP1 (or OPN), and then normal human serum. When the cells were assayed for viability by thiazolyl blue the domain(s) of DMP1 or OPN that interact with the CD44 protein on the surface of the MEL cells could be determined.

Treatment of MEL cells with normal human serum alone (GRGDS, SIBLING) resulted in significant cell death (Fig. 7A). Incubation of the cells with SIBLING (GRGDS, SIBLING) prior to the addition of normal human serum gave rise to cell protection. Pretreatment with GRGDS (GRGDS, SIBLING) alone had no effect on cell viability and was equivalent to control (GRGDS, SIBLING). Saturation with GRGDS did not entirely block the protective effect of OPN or DMP1 (GRGDS, SIBLING), consistent with OPN and DMP1 binding to the alternate receptor, CD44.

To investigate the sequences involved in SIBLING binding to CD44, experiments were carried out where the αvβ3 integrin was first saturated by an RGD-containing peptide, thus, fragments will only block activity if they contain regions involved in CD44 binding. When GRGDS saturation was followed by incubation with the various OPN and DMP1 fragments, the aminoterminal region of both clearly blocked the SIBLING's normal protective effect by 80 to 90% (Fig. 7B). With the exception of the DMP1 carboxyl terminus, the other fragments had little or no effect. Thus, the amino terminus of both OPN and DMP1 appear to be the major sites of interaction with CD44. Furthermore, they are similar to each other and distinct from that of BSP, a SIBLING which does not interact with CD44 in this functional assay. The structural sequence involved in CD44 binding was further refined by the use of synthetic peptides in a competition assay as described above. The peptides corresponding to exons 3 and 4 from osteopontin were screened for the ability to abolish DMP1- or OPN-conferred protection from alternative complement mediated cell lysis. The peptide corresponding to VKQADSGSSEEKQ significantly reduced DMP1 and OPN-mediated protection (Fig. 7C). The sequences that successfully blocked the protection from ACP-mediated lysis conferred by OPN or DMP are listed in Table I. Of the peptides tested, fragment 1 from OPN and DMP1 as well as DMP1 fragment 8 reduced cell survival. A conserved potential binding sequence among all three peptides is illustrated in Table I.
three separate experiments were carried out for DMP1 fragments. The average of all experiments by the thiazolyl blue assay (as in Fig. 4). Two separate experiments were carried out and combined for the OPN fragments and OPN peptides, while GRGDS (panel C). All conditions were then treated with a 1:10 dilution of normal human serum and after 2 h the cells were analyzed for viability by an incubation with 10 g/ml OPN exon 3 or OPN exon 4 peptide and then followed by an incubation with 5 g/ml OPN exon 4 of intact recombinant OPN or DMP1 and then a 1:10 dilution of normal human serum (+GRGDS +SIBLING) (panel A). For the fragment studies, MEL cells in GVB-MgEGTA buffer were treated with 400 nM GRGDS to saturate the αβ integrin, followed by an incubation separately with 8 different fragments of either OPN or DMP1 (numbers 1–5, at 10 μg/ml) and then followed by an incubation with 5 μg of intact recombinant OPN or DMP1 (panel B). For the peptide studies, MEL cells in GVB-MgEGTA buffer were treated with 400 nM GRGDS to saturate the αβ integrin, followed by an incubation with 10 μg/ml OPN exon 3 or OPN exon 4 peptide and then followed by an incubation with 5 μg of intact recombinant OPN or DMP1 (panel C). All conditions were then treated with a 1:10 dilution of normal human serum and after 2 h the cells were analyzed for viability by the thiazolyl blue assay (as in Fig. 4). Two separate experiments were carried out and combined for the OPN fragments and OPN peptides, while three separate experiments were carried out for DMP1 fragments. The average of all experiments ± S.D. is shown.

**TABLE I**

*SIBLING* sequences involved in CD44 binding

| Peptide sequence that blocks protective effect of intact SIBLING. |
|---------------------------------------------------------------|
| **DMP1–1** | LPVARYQNTESKSSEWKPQSLQQTPEPLLESEESSEKLSSEEQ |
| **DMP1–8** | EEDSHTLSHSSKRESQADESSSESSISQDHEPDEISSSQQZQLQSSISSAESQSESSHSEDSSSQDSSRSEDNSTSE |
| **OPN–1**  | IPVKCQDSQSGQKLYNKYPDAGATWLNPDPSQKQNLAPQTLPSKWSHDH |
| **Exon 3** | VQKADGSGSEQQ |

number of immune cells. To investigate which pathway is involved in the SIBLING/Factor H-mediated dampening of complement lytic activity, a series of experiments using the MEL cell line, recombinant adenovirus produced SIBLINGs and complement active normal human serum were carried out. In these experiments, the serum is pretreated with specific blocking antibodies prior to its addition to cells in the presence or absence of a given SIBLING. Two monoclonal antibodies against Factor I were used at different doses (1:4000, 1:2000, and 1:1000) in pretreating the human serum. One of these antibodies has been characterized as a "cleavage-blocking" antibody in that, when it is bound to Factor I, there is no cleavage of C3b. The other anti-Factor I antibody, when bound to Factor I, does not block C3b cleavage.

When DMP1 as well as OPN and BSP were assayed for Factor I involvement in SIBLING-conferred protection from complement, the cleavage blocking anti-Factor I antibody diminished the SIBLING’s ability to protect the cells (Fig. 8A). In contrast, the other non-function blocking antibody did not significantly alter the SIBLING’s ability to protect the cells. A dose response was evident in the ability of the cleavage-blocking antibody to inhibit cell protection by DMP1 (Fig. 8B). These results are consistent with a model where SIBLING-mediated protection from lysis involves Factor H action through Factor I. It is possible that some Factor H-mediated DAF activity is also present, however, it is insufficient by itself to stop ACP lysis. Thus, the (cell surface receptor (αβ) or CD44), -SIBLING- Factor H) complex acts similar to the membrane cofactor protein (MCP, CD46) in facilitating C3b degradation by binding and activating Factor I.

**DISCUSSION**

Complement plays a role in immune adherence, inflammation, opsonization, viral neutralization, localization of antigen, and cell lysis. The complement system can be activated by at least three distinct pathways: the classical pathway (usually involving immunoglobulins), the alternate pathway, and the lectin pathway (22). While the initiators of each pathway are different, all pathways converge in formation of the membrane-bound C3 convertases (Fig. 9). The different activation pathways employ different proteins to form the C3-convertases, however, all C3 convertases are multicomponent serine proteases that cleave the same single peptide bond in serum protein C3 generating two active fragments. A small peptide, C3a, is released as well as the major fragment C3b which can covalently attach to local targets. The newly bound C3b directs immune clearance, antigen selection, and cell lysis (19–22). C3b can also function via the ACP C3-convertase in an amplification loop generating more bioactive C3b. Thus, active complement results in the covalent attachment of a large number of C3b molecules clustered around the C3-convertase. C3b binding to C4b2b or C3bBb subunits of C3-convertase results in C5-convertase activity. C5-convertase acts on C5 generating C5a, a small peptide expressing anaphylotoxin and chemotaxin...
activity, and C5b, a large fragment that initiates assembly of the membrane attack complex.

Complement is regulated by a family of proteins, termed regulators of complement activation. The family of proteins include complement receptors one (CR1, CD35) and two (CR2: CD21), DAF (CD55), membrane cofactor protein (MCP, CD46), as well as Factor H (23). There are two major mechanisms by which Factor H and its cofactors can disrupt the lytic portion of the ACP. Factor H, when induced into a proper conformation by binding to other proteins or to certain carbohydrate groups, can participate in the formation of C3 convertase. Factor H has weak cofactor activity that can be greatly enhanced by binding to various factors.

Using MEL cells and recombinant DMP1, we have found that, following interaction with a specific cell surface receptors (α5β1 or CD44), DMP1 sequesters Factor H to the membrane phase and that this interaction quenches alternative complement-mediated cell lysis by normal human serum. Furthermore, DMP1 and OPN binding to CD44 was found to involve predominantly these two SIBLING’s amino-terminal region and could be blocked by an amino-terminal peptide. Finally, the receptor-SIBLING-Factor H complex clearly used functional Factor I in order to protect cells from lysis. These results suggest a shared biological activity between the SIBLING family members BSP, OPN, and DMP1 in their ability to regulate complement activity through Factor I-mediated cleavage of C3b (Fig. 9B). In solution phase, SIBLING binding to Factor H yielded no protection from lysis, while Factor H binding to SIBLINGs previously associated with the membrane phase (i.e. bound to α5β1 and/or CD44) resulted in protection from lysis. This biological activity of the SIBLINGs was found to include a MCP-like mechanism associated with the complement cofactor, Factor H. That all three SIBLING members studied, DMP1 (in the current study) and BSP and OPN (10) share Factor H binding suggests that common structural features are involved, such as polyacrylic amino acid sequences and high sialic acid content. It is of note that the presence of sialic acid on a cell surface increases the affinity of C3b for factor H, which prevents the formation of C3-convertase (24, 25).

The complement dampening activity of these three SIBLINGs is negated whenever the proteins are bound first by the very abundant Factor H (~0.5 mg/ml in serum) prior to binding to the appropriate cell surface receptor(s). This raises the interesting point that the range of this complement dampening activity must be relatively short. It seems reasonable to assume that only the cell actually secreting the protein (autocrine) or possibly cells within a short diffusional distance (paracrine) can bind the SIBLINGs to their cell surfaces and be protected from local complement activity. Cells expressing SIBLING family members and the appropriate cell surface receptors would have the capacity to locally dampen the complement cascade. In the case of complement and immune adherence, SIBLING expression might confer an immunoprotected status.-inflammatory responses involving complement may also be subject to SIBLING modulation. Increased OPN levels have been observed in wounds (26, 27), while elevated BSP levels have been observed in arthritis (28–30). Up-regulation of regulators of complement activation proteins has been observed in inflammatory tissues and organs affected by autoimmune diseases, while expression of regulators of complement activation components by autologous cells undergoing apoptosis was decreased (31). Expression of SIBLINGs by neoplasms (as has been seen for BSP and OPN) may provide a “gain-of-function” in a selective survival advantage for tumor cells (10). This survival advantage involves the new ability of the cancer cell to subvert the immune/complement system of surveillance through SIBLING sequestration of complement Factor H to the tumor cell surface. The membrane phase SIBLING-Factor H complex then recruits Factor I in the cleavage and clearance of C3b, thereby dampening complement activity and cloaking the cells from surveillance. The biological significance of the serum SIBLING-Factor H complex could be that it scavenges free
SIBLINGS and prevents systemic SIBLING-mediated complement regulation. From a biochemical standpoint, the significance of the serum complex is that it is necessary to disrupt the complex in order to measure total serum SIBLING levels. We have recently described the development of competitive immunoassays to measure total BSP and OPN in serum from normal donors and patients with various types of cancer (32). Disruption of the serum complex enabled BSP and OPN to be measured with a high degree of sensitivity and specificity.

The four acidic SIBLINGS (BSP, DMP1, dentin sialophosphoprotein, and OPN) are all often considered to be “matrix” proteins, largely because they accumulate in the mineralized matrices of bones and teeth. With respect to immunolocalization within the matrix, BSP and OPN are the two most extensively studied. Within bone, both appear to be enriched in the areas of de novo bone synthesis and the location where bone removal by osteoclasts has halted and a layer of collagen-poor matrix is made immediately prior to the formation of replacement bone (33–35). These areas are called cement (or reversal) lines and are where, first, an old matrix is exposed and then a new bone matrix is formed. One intriguing question is whether the SIBLINGS that accumulate there have any complement related activity. Most of the studies of complement involve understanding how cells such as bacteria or infected host cells are opsonized and/or lysed and how our normal cells escape this process. However, because the first step of the alternative pathway of complement is the spontaneous production of activated C3 and this activated C3 will form covalent linkages with various hydroxyl (OH) and amine (NH) groups on proteins and carbohydrates, it is reasonable to assume that exposed matrices will have activated C3 bound to them. Indeed, the subendothelial extracellular matrix in vitro becomes labeled with C3 when the lining endothelial cells retract (36). In the case of the mineralized matrices and, it is also known that hydroxyapatite is one of the substances that can directly activate the classical pathway of complement (without the contribution of immunoglobulins) (23), thus adding another possible method of locally activating the complement cascade in these tissues.

It is widely assumed that the OH and NH groups of matrices are not as good receiving groups for the activated C3 as are bacteria, infected cells etc., and as such do not accumulate large amounts of complement. Even if this is so, the long period of exposure of some matrices to normal complement (some matrices last for many years), and the lack of the membrane-bound DAF and MCP-like activities available to cells would ensure an accumulating level of at least the more stable NH-bound C3. In light of our recent data, it is intriguing to consider that the accumulation of the SIBLINGS at the very sites of old or new matrix may suggest that these proteins are involved in both quenching the ACP on cells in the immediate vicinity of matrix exposure and also, through interaction with Factors H and I, result in the destruction of any C3 accumulating on the local matrices.

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