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Regulation of Complement Classical Pathway by Association of C4b-Binding Protein to the Surfaces of SK-OV-3 and Caov-3 Ovarian Adenocarcinoma Cells

Mikko T. Holmberg,* Anna M. Blom, † and Seppo Meri2‡

The role of fluid-phase regulators of complement is to inhibit excessive complement activation and maintain homeostasis in blood. To prevent excessive activation, the complement system is regulated by fluid-phase and membrane-bound proteins. Complement activation on cell surfaces is mainly controlled by membrane regulators, which include protectin (CD59), decay-accelerating factor (DAF), CD35, membrane cofactor protein (MCP), C4b-binding protein (C4bp), and CR1 (CD35). CD59 regulates the terminal pathway by inhibiting formation of the membrane attack complex (1). MCP and CR1 act at the earlier steps of complement activation in both the classical and alternative pathway by acting as cofactors for factor I (fI)-mediated cleavage of C4b and C3b to their inactive products (2, 3). Because C4b and C3b are subunits of the C3/C5 convertase enzyme complex (C4b2a in the classical, and C3bBb in the alternative pathway) in the complement activation cascades, MCP and CR1 thereby limit the number of convertases formed. DAF and CR1 promote directly the dissociation of the catalytic subunits C2a and Bb from the convertases (4, 5). The tissue distribution of CD59, MCP, and DAF is wide. These proteins are present on most circulating cells, endothelia, and on epithelial and mesenchymal cells of many organs. CR1 is found mostly on circulating cells, glomerular podocytes, and on cells of leukocytic origin in tissues.

Fluid-phase inhibitors of complement that control the C3/C5 convertases include factor H (fH), fH-like protein 1, and C4b-binding protein (C4bp). C4bp has a similar decay-accelerating activity as DAF on the C3/C5 convertase of the classical pathway, and it can act as a cofactor for fI-mediated cleavage of C4b (6, 7). fH and fH-like protein 1 have analogous functions in regulating the C3bBb enzyme complex (8–10). The primary role of these soluble complement regulators is to prevent excessive activation and depletion of complement in the fluid phase.

C4bp is a large plasma protein with an apparent molecular mass of 540–590 kDa in SDS-PAGE. It seems to be synthesized only in liver and is secreted to plasma to maintain an average concentration of 160 μg/ml. About half of C4bp in blood is bound to the anticoagulant protein S. This binding blocks the anticoagulant activity of protein S, but has no known effect on the complement-regulatory activity of C4bp (11). As protein S associates with negatively charged lipid membranes, it has been proposed that this interaction could direct C4bp to sites of cell injury on cell surfaces and thus protect tissues from excessive complement activation. C4bp is composed of six or seven 70-kDa α-chains and one or no 45-kDa β-chain, depending on the isoform (12). Electron microscopic analysis of C4bp has revealed a spider-like structure, in which the α-chains and the β-chain are linked together in the central core by their carboxyl-terminal domains (13). The α-chains consist of eight domains called either short consensus repeats or complement control protein (CCP) units. These domains are common to all regulators of complement activation, whose genes are encoded in the long arm of chromosome 1 (14, 15). In C4bp, the amino-terminal CCP1 and CCP2 in the α-chain are involved in the binding of C4b and are critical for the functional activity (16). As a cofactor for fI, C4bp promotes cleavage of the C4b molecule to C4c and C4d, thus preventing formation of the C4b2a enzyme complex. C4b is composed of α (93 kDa), β (75 kDa), and γ-chains (32 kDa) linked together with disulfide bonds. The fI-mediated cleavages occur in the α-chain between R277 and T938.
and between R1317 and N1318. The fragment between these cleavage sites is C4d (45 kDa), and the remaining part of the molecule is called C4c (6, 17, 18).

Previously, we have observed that malignant glioblastoma cells (the H2 cell line) can bind the soluble complement alternative pathway regulator FH directly to their cell surfaces and thus inhibit complement activation (19). This mechanism is actually so efficient that even if all the known membrane complement regulators were blocked with mAbs, little or no lysis of the H2 cells could be observed after treatment with complement-activating Abs and serum. This was due to the fact that FH bound to the surface of H2 cells restricted the activation cascade at the C3 level. The principal mechanism whereby the fluid-phase regulators can protect cells from complement attack involves first, the binding of the regulator to C3b or C4b that have become covalently bound to the cell surface, and second, the reactions leading to the inactivation of the C3 convertases (cofactor and decay-accelerating functions). However, the ability of a regulator to directly bind to a cell surface can significantly increase the efficacy of complement inhibition, as the cell may become coated with a fluid-phase regulator also in the absence of initial complement activation. It has been demonstrated previously that in addition to H2 cells, FH can bind directly to a variety of other cells (20–22). Surface polyansions such as sialic acid have been shown to be important in promoting FH binding to cells, particularly after an initial deposition of C3b. Surface-bound FH thus directs subsequent events toward inhibition of complement activation (23–25).

In the present study, we screened malignant cell lines to find out whether also C4bp, a functional analogue of FH in the classical pathway, could bind to cell surfaces. As a model, we present SK-OV-3 and Caov-3 ovarian adenocarcinoma cell lines. We observed that C4bp binds to these cells via α-chain domains that are not involved in the complement-regulatory activity. Demonstration of C4bp binding to these cells in a functionally active form establishes a novel mechanism whereby human tumor cells can regulate complement activation.

Materials and Methods

Proteins and other reagents

Human C4bp was purified from plasma, as described previously (26). Protein S was removed from the molecule during purification. Polymeric recombinant mutant C4bp molecules lacking individual short consensus repeat domains were constructed and expressed essentially as described previously (27). The mutants, denoted as ΔCCP1, ΔCCP1–2, ΔCCP2, ΔCCP3, ΔCCP4, ΔCCP5, and ΔCCP6, lack either CCP1, both -1 and -2, -2, -3, -4, -5, or -6, respectively. Human C4b and FI were obtained from Calbiochem (La Jolla, CA). C4bp, the deletion mutants, and C4b were labeled with 125I by the Iodogen method (28). A PD-10 gel filtration column (Amersham Pharmacia Biotech, Uppsala, Sweden) was used for separation of free iodine from the labeled protein, and Veronal-buffered saline (pH 7.4) was used as a buffer. Purities of the labeled proteins were verified by SDS-PAGE and autoradiography. Four mAbs against the α-chain of C4bp were used. mAbs 104 and 96 recognize CCP1–2, mAb 102 is against CCP4, and mAbs 67 and against CCP2. These Abs have similar affinities for the C4bp were used. mAbs 104 and 96 recognize CCP1–2, mAb 102 is against CCP4, and mAbs 67 and against CCP2. These Abs have similar affinities for the

C4bp BINDING TO OVARIAN TUMOR CELLS

Binding of radiolabeled C4bp to cells

To analyze the functional activity of cell-bound C4bp, its cofactor activity for FH-mediated cleavage of 125I-labeled C4b (125I-C4b) was tested. For C4bp binding, 3 × 10⁵ cells were incubated with C4bp at a 50 μg/ml concentration in 300 μl RPMI 1640 medium (no FCS added) for 40 min at 37°C. The cells were then washed twice with PBS (pH 7.4) and divided into separate microtubes to have 3 × 10^⁶ cells/tube. To block the cofactor activity of cell surface MCP and/or C4bp, GB24 and mAb104 Abs, respectively, were added to the appropriate samples at a concentration of 10 μg/ml and incubated for 15 min at 37°C. Factor I (17 μg/ml) and 125I-C4b (6 μg/ml) were added, and the samples were incubated in a final volume of 60 μl for 100 min at 37°C. A positive control sample for C4bp degradation contained 10 μg/ml C4bp, 17 μg/ml FI, and 6 μg/ml 125I-C4b in RPMI 1640. The negative control had 17 μg/ml FI and 6 μg/ml 125I-C4b in RPMI 1640. After incubation, the cell samples were centrifuged and the supernatants were subjected to SDS-PAGE analysis under reducing conditions. In addition to the actual cofactor activity test, control samples for each cell line were prepared to monitor for possible cofactor activity of C4bp dissociated from the cells during the Ab and 125I-C4b/II incubations. These samples were treated similarly as the other samples until the 125I-C4b/II incubation step. However, at this point, no 125I-C4b or FI was added, but the samples were incubated in RPMI 1640 with 33 μg/ml GB24 for 100 min under similar conditions as the actual test samples. The tubes were then centrifuged, and the supernatants of the control samples were removed and mixed with 125I-C4b (6 μg/ml) and FI (17 μg/ml), and incubated for another 100 min.

Results

Binding of 125I-C4bp to cells

To find out whether tumor cells could bind C4bp from the fluid phase, binding experiments with radiolabeled C4bp were performed. Of the various cell lines tested, SK-OV-3 adenocarcinoma cells bound C4bp the most, and PA-1 cells the least. Caov-3 and SW626 cells bound intermediately (Fig. 1). To further examine the nature of the binding, inhibition tests with unlabeled C4bp and BSA were performed. 125I-C4bp binding to cells was inhibited by 40% when unlabeled C4bp was added within a concentration range of 80–120 μg/ml into a sample buffer that contained 0.3 μg/ml 125I-C4b. Under similar conditions, 100–500 μg/ml BSA had no significant effect (0–10% inhibition of binding). The binding of C4bp to cells was sensitive to an increasing salt concentration in the sample buffer. The binding of 125I-C4bp increased 6-fold in GVBS containing 70 mM NaCl, and decreased by 50% at 400 mM, when compared with the binding in GVBS containing a physiological concentration of NaCl (150 mM).

Mapping the cell binding site on C4bp with Abs

Inhibition tests with Abs were performed to tentatively map the binding site on C4bp. Different concentrations of either polyclonal Abs or mAbs against C4bp were added to the sample buffer with 125I-C4bp and incubated for 15 min at 20°C before the cells were
Inhibition of C4bp binding to cells with anti-C4bp Abs. (A) Increasing amounts of polyclonal Abs against C4bp α- or β-chain were incubated in GVBS with 125I-C4bp and SK-OV-3 cells. The binding results are expressed as mean ± SD relative to binding in samples without Abs. The addition of the anti-C4bp α-chain polyclonal Ab inhibited 125I-C4bp binding to cells, whereas the β-chain polyclonal Ab induced a slight increase in the binding. (B) Effect of mAbs against C4bp α-chain on 125I-C4bp binding to SK-OV-3 cells. The mAbs 102, 96, and 67 bind to CCPs 1, 1–2, and 4, respectively. All three mAbs inhibited 125I-C4bp binding, but mAb 67 (anti-CCP4) caused the strongest inhibition. Mean ± SD values are from two different experiments with duplicate samples.

Mapping the cell binding site on C4bp with rC4bp mutant proteins

To further characterize the interaction between C4bp and SK-OV-3 cells, a set of 125I-C4bp α-chain deletion mutants was tested in the binding experiments (Fig. 3). These tests were performed with 90 mM NaCl in the sample buffer. The binding of ΔCCP1 and ΔCCP6 to cells was as strong or even slightly stronger than that of the wild-type C4bp (6% binding of offered 125I-C4bp). The mutants that had CCP2, -3, -5, or both -1 and -2 removed bound immediately (2–3% binding). The only mutant that bound clearly more weakly than any other protein was ΔCCP4 (0.8% binding). This suggests that CCP4 is the primary cell surface binding site on C4bp, which is in accordance with the mAb inhibition tests.

Cofactor activity of cell-bound C4bp

To analyze whether binding of C4bp had any functional consequences, cofactor activity tests for fI-mediated C4b degradation were performed with C4bp-preincubated and control cells. The cofactor activity of cell surface MCP (CD46) was blocked with the GB24 mAb. In these experiments, SK-OV-3, Caov-3, and SW626 cells induced fI-mediated cleavage of 125I-C4b in the sample solution, also in the presence of GB24. This was seen in SDS-PAGE analysis of the sample supernatants as a characteristic fI-mediated cleavage pattern of C4b (Fig. 4A). In the samples that contained both GB24 and mAb104 that blocks the cofactor function of C4bp, no cleavage of 125I-C4b was observed. PA-1 cells, preincubated with C4bp, had a weaker cofactor activity than the other cell lines, and it was completely inhibited by GB24 alone. To find out whether the cofactor activity seen in these cell samples was actually caused by cell-bound C4bp and not by C4bp that had become dissociated from the cells during the experiment, the supernatants of control cell samples were tested for their cofactor activity. SK-OV-3 and Caov-3 cells did not release any cofactor activity for C4b inactivation to the supernatants, whereas the SW626 cell supernatants had some cofactor activity (Fig. 4B).

Discussion

The present study shows that C4bp can bind to malignant ovarian tumor cells via its α-chain without prior complement activation.

FIGURE 1. Binding of 125I-C4bp to ovarian tumor cell lines. Cells were incubated with radiolabeled C4bp in GVBS with 150 mM NaCl. 125I-C4bp concentration ranged from 0.1 to 2 μg/ml. The results are expressed as the amount of 125I-C4bp bound to 2 × 10⁶ cells (mean values ± SD from three experiments with duplicate samples in each). SK-OV-3 cells were the strongest binders of C4bp, Caov-3, and SW626 cells bound intermediately, and with the PA-1 teratocarcinoma cell line the 125I-C4bp binding was weak.

FIGURE 2. Inhibition of C4bp binding to cells with anti-C4bp Abs. A, Increasing amounts of polyclonal Abs against C4bp α- or β-chain were incubated in GVBS with 125I-C4bp and SK-OV-3 cells. The binding results are expressed as mean ± SD relative to binding in samples without Abs. The addition of the anti-C4bp α-chain polyclonal Ab inhibited 125I-C4bp binding to cells, whereas the β-chain polyclonal Ab induced a slight increase in the binding. B, Effect of mAbs against C4bp α-chain on 125I-C4bp binding to SK-OV-3 cells. The mAbs 102, 96, and 67 bind to CCPs 1, 1–2, and 4, respectively. All three mAbs inhibited 125I-C4bp binding, but mAb 67 (anti-CCP4) caused the strongest inhibition. Mean ± SD values are from two different experiments with duplicate samples.

FIGURE 3. Binding of C4bp deletion mutants to SK-OV-3 cells. Radiolabeled deletion mutants lacking individual CCP domains were allowed to bind to 2 × 10⁶ cells in GVBS with 90 mM NaCl. As a control, the binding of plasma-purified 125I-C4bp was tested under similar conditions. The results are expressed as mean amount of 125I-C4bp per mutant bound to cells plotted against amounts of protein offered in the sample. ΔCCP1 and ΔCC6 bound as well as purified C4bp. ΔCCP4 bound only in very low quantities, confirming the importance of CCP4 to the interaction of C4bp and cells. All the other mutants bound with an intermediate efficiency.
and C4b deposition. The primary binding site on C4bp was found to be on CCP4 of the $\alpha$-chain. The cofactor activity of cell-bound C4bp for C4b inactivation was retained, suggesting that this phenomenon directs complement-regulatory activity to the cell surfaces. Direct binding of C4bp to cell surfaces has not been reported before, and this finding suggests that in ovarian tumor tissue C4bp can bind to cell surface structures and lead to an increased control of the classical pathway activation.

Of the cell lines tested, the binding of C4bp was strongest to the SK-OV-3 adenocarcinoma cells. These cells bound appreciable amounts of C4bp under physiological ionic strength conditions. The other ovarian adenocarcinoma cells, Caov-3 and SW626, also bound C4bp, whereas with the PA-1 teratocarcinoma cells, the binding was negligible. The binding of C4bp to cells was sensitive to salt concentration in the sample buffer, suggesting that the interaction is of an electrostatic rather than of hydrophobic nature. Relatively high concentrations of unlabeled C4bp were required in the inhibition tests to compete out $^{125}$I-C4bp binding to cells. This suggests a large number of binding sites on cell surface, and in contrast, a possibly relatively low affinity interaction. Initial ligand blotting experiments with $^{125}$I-C4bp have not revealed any protein ligand on the cell membranes (data not shown). Together these results suggest that the cell surfaces have diffusely distributed acceptors, such as carbohydrates or proteoglycans, which carry a large reservoir of negative charge, a possible attractant of C4bp molecules.

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The cell surface binding sites on C4bp were found to be on CCP4 of the $\alpha$-chain, as the polyclonal $\alpha$-chain-specific Ab inhibited the binding, but the anti-\(\beta\)-chain Ab did not. Both the inhibition tests with mAbs and the binding tests with C4bp $\alpha$-chain deletion mutants indicated that CCP4 of the $\alpha$-chain is the most important domain for the binding. Removal of CCP1 or CCP6 did not interfere with the C4bp binding, ruling out these domains as possible binding sites. The small inhibiting effect of the mAb 102 (anti-CCP1) could have been due to steric hindrance. The other deletion mutants bound intermediately, making it difficult to judge the importance of CCP2, -3, and -5 for the binding. It is possible that these neighboring domains also contribute to the binding. In contrast, in deletion mutants, one cannot exclude changes in interdomain structure that may affect C4bp binding to cells. In conclusion, while multiple interactions between the cell surface and C4bp

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**FIGURE 4.** Cofactor activity test for cell-bound C4bp. A, Four indicated tumor cell lines were incubated with purified C4bp (50 \(\mu\)g/ml), washed carefully, and allowed to react with $^{125}$I-C4b and \(\Phi\) to see whether the cell-bound C4bp retained its cofactor activity. The experiments were conducted in duplicate, either in the absence of Abs, or in the presence of GB24 (anti-MCP mAb) or both GB24 and mAb 104 (anti-C4bp) in the sample. In addition to cell samples, controls for \(\Phi\)-mediated C4b cleavage were examined. The positive control (+) had \(\Phi\) and purified C4bp with $^{125}$I-C4b. The negative control (−) had only \(\Phi\) with $^{125}$I-C4b. In the absence of mAbs, all the cell lines promoted C4b degradation. This can be seen as the appearance of the physiological cleavage fragments C4d (45 kDa) and \(\alpha_4\) (15 kDa) in the SDS-PAGE run under reducing conditions. SK-OV-3, Caov-3, and SW626 cells retained cofactor activity under conditions in which the function of MCP was blocked with GB24. As the remaining cofactor activity could be blocked with the anti-C4bp mAb, the MCP-independent cofactor activity seen on these cells must be due to C4bp. The 50- to 60-kDa band seen in all samples is a nonspecific fragment, present in the original C4b batch, and should not be mixed with the 45-kDa C4d band. B, To confirm that the cell-bound C4bp was actually the cofactor in these tests, and not C4bp dissociated from the cells to the sample supernatant during the incubation, control experiments were performed. A duplicate set of cells was preincubated with C4bp and the $\alpha$-MCP mAb GB24 and washed. After a 100-min incubation, the supernatants of the samples were removed and tested for C4bp-dependent cofactor activity by adding $^{125}$I-C4b and \(\Phi\). Neither the SK-OV-3 nor the Caov-3 supernatants showed any cofactor activity, suggesting that C4bp remained associated with the cells during the experiment. In contrast, the supernatants of SW626 cells showed some cofactor activity, which can be seen as the appearance of the 45-kDa C4d and 15-kDa \(\alpha_4\) fragments.
α-chain CCPs 2 to 5 may exist, the CCP4 domain has the strongest activity.

The functional activity of cell-bound C4bp as a complement regulator was analyzed by testing its cofactor activity for F-mediated inactivation of C4b. Cofactor activity tests showed that after binding of purified C4bp, SK-OV-3, Caov-3, and SW626 cells could degrade C4b under conditions in which the cofactor activity of MCP was blocked by the GB24 Ab. As all the cofactor activity for C4b degradation could be blocked by adding both GB24 and the anti-C4bp mAb104 to the sample buffer, the other cofactor in these samples must have been C4bp, and no other cofactors were present. To confirm that cell-bound C4bp was actually the principal cofactor and not free C4bp released into the sample supernatant, additional experiments were performed. The results did not show any cofactor activity in the supernatants of other than the SW626 cells. This suggests that with SK-OV-3 and Caov-3 cells, the binding interaction was strong enough to keep most C4bp associated with cells during the experiment, and that cell-bound C4bp retained its cofactor activity. With SW626, it is difficult to determine whether the cell-bound C4bp or dissociated C4bp is actually responsible for the cofactor activity. The results of the cofactor tests are in agreement with the binding results, as the release of cofactor activity to the SW626 sample supernatant suggests a weaker affinity of C4bp binding. Also, the PA-1 cells did not recruit any C4bp-mediated cofactor activity at all, in accordance with the weak binding of C4bp.

From the physiological point of view, the most obvious implication of C4bp binding to cells is an increased complement-regulatory activity on the cell surfaces. As C4bp is a regulator of the classical pathway C3 convertase, which acts early in the cascade, it can effectively control most of the complement activation events, classical pathway C3 convertase, which acts early in the cascade, and P. J. Lachmann. 1990. Human protectin (CD59), an 18,000–20,000 MW complement lysis restricting factor, inhibits C5b-8 catalyzed insertion of C9 into cell membranes. J. Immunol. 145:569.

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References


