Published in final edited form as:
*Eur J Immunol*. 2013 June ; 43(6): 1617–1629. doi:10.1002/eji.201243196.

# Analysis of genes coding for CD46, CD55 and C4b-binding protein in patients with idiopathic, recurrent, spontaneous pregnancy loss

**Frida C. Mohlin**[1], **Eric Mercier**[2], **Veronique Fremeaux-Bacchi**[3], **M. Kathryn Liszewski**[4], **John P. Atkinson**[4], **Jean-Christophe Gris**[2], and **Anna M. Blom**[1]

[1]Department of Laboratory Medicine, Lund University, Malmö, Sweden

[2]Laboratory of Hematology, University Hospital, Nimes, France

[3]Service d'Immunologie Biologique, Hopital Europeen Georges Pompidou, Paris, France

[4]Department of Medicine, Division of Rheumatology, Washington University School of Medicine, St. Louis, MO, USA

## Summary

Since a tightly regulated complement system is needed for a successful pregnancy, we hypothesized that alterations in complement inhibitors may be associated with idiopathic, recurrent miscarriage. We sequenced all exons coding for three complement inhibitors: C4b-binding protein (C4BP), CD46 and CD55 in 384 childless women with at least two miscarriages that could not be explained by known risk factors. Several alterations were found in *C4BPA*, of which the R120H, I126T, and the G423T mutations affected the expression level and/or the ability of recombinant C4BP to serve as cofactor for factor I. The only variant in *C4BPB* was located in the C-terminal part, and did not impair the polymerization of the molecule. Our results identify for the first time alterations in C4BP in women experiencing recurrent miscarriages. We also found four *CD46* alterations in individual patients that were not found in healthy controls. One of the rare variants, P324L, showed decreased expression, whereas N213I resulted in deficient protein processing as well as an impaired cofactor activity in the degradation of both C4b and C3b. The identified alterations may result in in vivo consequences and contribute to the disorder but the degree of association must be evaluated in larger cohorts.

### Keywords

complement system; complement inhibitor; mutation; reproductive immunology

## Introduction

Spontaneous pregnancy loss is a common complication of pregnancy, affecting 15% of clinically recognized pregnancies and almost 50% of all pregnancies. Recurrent spontaneous pregnancy loss (RSPL), defined as three or more consecutive losses, affects approximately 1% of couples trying to conceive [1]. The most common causes of RSPL are genetic,

**Corresponding author**: Anna Blom, Lund University, Department of Laboratory Medicine Malmö, The Wallenberg Laboratory floor 4, SE-205 02 Malmö, Sweden. Tel. +46 40 33 82 33; fax: +46 40 33 70 43; anna.blom@med.lu.se.

**Conflict of interest**
The authors declare no financial or commercial conflict of interest.

structural, autoimmune and endocrine abnormalities, together with infections and thrombophilic disorders but approximately 40% of the cases still remain idiopathic [1].

The role of the complement system in physiological and pathological pregnancy is complex but unequivocal [2]. Complement is a pivotal part of innate immunity and not only protects the host from infections but also participates in many processes to maintain tissue homeostasis. Complement-mediated damage to autologous tissues is prevented by inhibitors typically consisting of complement control protein (CCP) domains. A fully active complement system, derived from the maternal circulation, as well as from local production by various cell sources, is present in the placenta [2]. Fetal tissues are semi-allogeneic and commonly cause development of alloantibodies. Therefore, the placenta is potentially subject to complement-mediated immune attack at the feto-maternal interface, with the potential risk of fetal loss. Excessive complement activation in the placenta places the fetus at risk for growth restriction or death. In a normal, successful human pregnancy undesirable complement activation is prevented by inhibitors, such as CD46 and CD55, expressed on the surface of the trophoblast [2]. The critical role played by these regulators is supported by the embryonic lethality observed in mice deficient in the complement inhibitor Crry [3], which resembles human CD46.

C4BP controls the classical and lectin pathways as it acts as a cofactor for the serine proteinase factor I (FI) in degradation of the activated complement components C4b/C3b and enhances decay of complement convertases. The major form of C4BP consists of seven identical α-chains and a unique β-chain [4]. The α- and β-chains contain eight and three CCP domains, respectively, and are encoded by two separate genes, *C4BPA* and *C4BPB*. C4BP, in complex with the anticoagulant protein S, binds strongly to apoptotic [5] and necrotic cells [6] and limits complement activation on these cells. To date, no individuals with inherited C4BP deficiency have been identified and we speculated that such defect may be involved in pregnancy loss due to the role of C4BP in apoptosis. Furthermore, *C4BPA* expression is normally up-regulated during the implantation window period but in women with unexplained RSPL, *C4BPA* was expressed at lower levels, compared with fertile controls [7]. Widely expressed, membrane bound CD46 acts as a cofactor for FI to inhibit all pathways of complement by inactivating C4b and C3b. Four major isoforms (BC1, BC2, C1 and C2) are expressed through alternative splicing. Isoforms vary in their quantity of O-glycosylation, as a result of the presence or absence of region B and in the expression of one of two possible cytoplasmic tails (Cyt-1 or Cyt-2). All isoforms contain four CCP domains that harbor complement inhibitory activity. Restriction fragment length polymorphism analyses for *CD46* indicated association with RSPL [8, 9] and recently, *CD46* alterations were identified in preeclampsia [10]. CD55 inhibits complement by dissociating the classical and alternative pathway convertases. The mature protein consists of four CCP domains and it is bound to the membrane via a glycosylphosphatidylinositol anchor. CD55 is present on the trophoblast [11] and it is down-regulated on the endometrium of women with the antiphospholipid syndrome [12].

Due to the role of complement in the physiology and pathology of pregnancy, we hypothesized that mutations or polymorphisms in complement inhibitors C4BP, CD46 and CD55 may be associated with pregnancy loss and herein report sequencing of all coding exons of these genes in women experiencing idiopathic RSPL.

## Results

### Mutations and polymorphisms identified in *C4BPA, C4BPB, CD46* and *CD55*

All exons of *C4BPA* and *C4BPB* were sequenced in 384 patients with RSPL and one heterozygous, non-synonymous mutation was found in the C-terminus of *C4BPB*, together

with several alterations in the signal peptide and in different CCP domains of *C4BPA* (Fig. 1A–C and Table 1). A few previously identified polymorphisms were also found in *C4BPA*, one (R240H) of which had been shown previously to impair the ability of C4BP to act as cofactor to degrade C3b [13].

Analysis of all *CD46* exons in 384 patients revealed five heterozygous, non-synonymous alterations in the coding exons (Fig. 1D and Table 2). Two of these were located in CCP3 (P193S and N213I), one in the serine/threonine rich region C (STP-C) close to the membrane (P324L) and one in Cyt-1 (T383I). We also found one previously identified polymorphism in the transmembrane domain, A353V. This polymorphism, which we found in 1.04% of the patients and 3.1% of the controls, has been shown to affect the ability of CD46 to control the alternative pathway activation [14].

Clinical data, regarding the patients carrying alterations in CD46 and C4BP, which alter expression or function are listed in Table 3. None of the alterations found in *CD46* and *C4BPA*/*C4BPB* were statistically significantly associated with the disorder, individually or combined, as determined by Fisher's exact test. However, this exploratory study has very limited statistical power to detect associations for rare mutations. Tables 1 and 2 provide the frequencies of currently identified alterations in two different databases (dbSNP and NHLBI Exome Sequencing Project). These data further indicate that the found mutations are very rare. Thus great emphasis was put on the functional characterization of the detected variants. Notably, four of the five alterations in *CD46* and four in *C4BPA*/*C4BPB* were only found in patients and not in healthy controls with at least two uneventful pregnancies. After sequencing of all coding exons in 192 patients, no non-synonymous alterations were found in *CD55*.

## Expression and characterization of C4BP α-chain variants

To determine if the C4BP α-chain alterations identified in the patients changed the expression, secretion or function of C4BP, seven of the found α-chain variants (P4Q, R120H, I126T, I224T, D284E, I300T and G423E) were expressed recombinantly. Transient transfections revealed that I126T and G423E were expressed at statistically significantly but only slightly decreased levels compared to WT, whereas R120H showed a modestly increased expression (Fig. 2A). These mutants were also expressed in a stable manner and then the protein was purified using affinity chromatography followed by SDS-PAGE analysis under both non-reducing (Fig. 2B) and reducing conditions (Fig. 2C). All proteins migrated with the same apparent velocity and were correctly assembled into a high molecular weight complex, containing six α-chains (Fig. 2B). One alteration, P4Q, was located in the signal peptide, which potentially could alter the processing of the protein. However, the N-terminal sequencing showed that the recombinant P4Q mutant had been processed at the same site as WT (data not shown).

## The cofactor activity of C4BP α-chain variants in C4b and C3b degradation

To test the function of the C4BP α-chain variants, degradation assays were performed, testing C4BP's cofactor activity to FI, in the degradation of C4b and C3b in fluid phase and also C3b degradation on the cell surface. In the degradation of C4b, R120H showed a significantly increased cofactor activity at both concentrations tested, whereas I126T showed a decreased activity (Fig. 3A–B). R120H and I126T demonstrated significantly decreased cofactor activity in the degradation of C3b, both in fluid phase (Fig. 3C–D) and on the cell surface (Fig. 3E–F). I224T also had a modestly impaired activity for C3b degradation in solution, for one of the tested concentrations (Fig. 3C–D).

### Expression and characterization of C4BP β-chain variant

The T232A mutation in the β-chain of C4BP was located in the C-terminus, which is responsible for the polymerization of the β-chain to the α-chains. To elucidate whether the mutation caused impaired polymerization, we expressed C4BP α- and β-chains simultaneously. After transient transfection, the total amount of C4BP, as well as only β-chain containing C4BP, in cell media and lysates were detected with an ELISA using specific antibodies for the α- and β-chain, respectively. Comparable levels of both C4BP α- (Fig. 4A) and β-chain (Fig. 4B) for WT and T232A were found in both cell media and lysates, indicating normal expression, secretion and polymerization of the β-chain T232A mutant.

### Expression and characterization of CD46 variants

In order to elucidate whether the CD46 mutations in the patients altered either the expression or the function of the protein, the mutants were expressed transiently on the surface of CHO cells. Flow cytometry analysis of the gated main cell population (Fig. 5A) revealed that one rare variant, P324L, showed a decreased expression on the cell surface at the level of ~55% compared with that of WT (Fig. 5B–C), together with decreased protein concentration in the cell lysate, using ELISA (Fig. 5D). N213I did not show significantly lower cell surface expression (Fig. 5B–C) but the protein concentration in the cell lysate was significantly decreased (Fig. 5D). Western blot analysis of the cell lysates demonstrated that all CD46 mutants migrated with the expected apparent mobility (Fig. 5E). However, in the cell lysate of the N213I mutant, a weaker band at 40 kDa was also observed. The molecular mass was similar to the precursor form of CD46, which normally corresponds to ~1% of the mature form and is usually not observed, unless the blot is overexposed [15]. The mature protein contains sialic acid residues, O-linked oligosaccharides and complex N-linked oligosaccharides, but the pre-Golgi CD46 precursor only carries N-linked high-mannose oligosaccharides [16]. After analyzing cell lysates from three separate transfections, we observed that the ratio between precursor and mature protein was increased to ~30% for N213I, compared with ~5% for WT and the other mutants (Fig. 5F). This suggests that N213I is processed less efficiently than WT.

### Binding of CD46 to C4b and C3b

The ability of the CD46 variants to bind to their ligands C4b and C3b was tested in a direct binding assay. First, the CD46 concentration in cell lysates from three independent transfections was determined using ELISA, in which increasing concentrations of CD46 were added to a plate coated with C4b or C3b. Cells transfected with empty pSG5 vector did not show CD46 expression (Fig. 5D) and corresponding volumes of this cell lysate were used as a negative control. All mutants were able to bind C4b (Fig. 6A) and C3b (Fig. 6B) equal to WT. T383I demonstrated a slightly increased binding to C4b for one of the tested concentrations.

### The cofactor activity of CD46 in C4b and C3b degradation

Next, we examined whether the analyzed mutations in CD46 impaired the cofactor function to FI in the degradation of C4b and C3b. Cell lysates, with known CD46 concentrations, were mixed with FI and C4b or C3b and incubated for different time points. Cells transfected with empty pSG5 vector did not express CD46 (Fig. 5D) and corresponding volumes of this cell lysate were used as a negative control. N213I showed decreased cofactor activity in both the degradation of C4b (Fig. 6C–D) and C3b (Fig. 6E–F).

## Discussion

Miscarriage is a common complication of pregnancy with approximately 40% of cases remaining idiopathic. Since embryonic/fetal tissues are semi-allogenic, there is a potential risk of pregnancy loss due to complement-mediated immune attack at the trophoblastic maternal interface. Therefore, we set out to determine if maternal mutations and polymorphisms in three complement inhibitors, CD46, C4BP and CD55, are associated with RSPL.

Number of alterations found in this study in *C4BPA/B* and *CD46* were predicted to have probably damaging effect on the resulting proteins by PolyPhen software and we have investigated this experimentally using recombinant proteins carrying identified mutations. Several alterations were found in *C4BPA*, of which two were located in the signal sequence and found both in patients and controls. Analysis of recombinant C4BP α-chain carrying the P4Q polymorphism showed that it neither affected the processing of the protein into a mature, secreted polymer nor the expression level. The I126T mutation in CCP2 of C4BP α-chain is of particular interest as it was found only in one patient but not in healthy controls. This rare mutation affected both expression level of C4BP α-chain as well as its function, i.e., degradation of C4b and C3b in solution. We showed previously that CCP2 is crucial for the ability of C4BP to interact with both C4b [17, 18] and C3b [19] and to present these for degradation by FI. Taken together, this could potentially cause a sufficient decrease in functional C4BP to mitigate in vivo consequences. The second mutation in CCP2 of C4BP α-chain, R120H, found in two patients and no controls, increased the ability of C4BP to act as cofactor in degradation of C4b but decreased its activity in degradation of C3b both in solution and deposited on the cell surface. Even though C4BP does not inhibit the alternative complement pathway nearly as efficiently as factor H, this defect in C3b degradation could decrease the extra protection provided by C4BP. A number of diseases are associated with dysregulation of the alternative pathway. The I224T in CCP3 of C4BP α-chain, found both in patients and controls, showed a slightly decreased cofactor function in the degradation of C3b, only in solution. The conserved D284E mutation, found in one patient, did not have any effect on the expression or function of C4BP implying that it is a functionally inert polymorphism. The R240H polymorphism in CCP4 of C4BP α-chain has been identified previously in atypical hemolytic uremic syndrome (aHUS) patients [13] and shown to affect the ability of C4BP to act as cofactor in degradation of C3b. In the current study, it was present in similar frequency in patients and controls indicating that it is not likely of importance for the analyzed disease. The I300T is a very frequent polymorphism, which did not affect expression or function of C4BP. Finally, the G423E in CCP6 of C4BP α-chain was found both in patients and controls affected the expression level of the protein modestly. The T232A mutation was found only in one patient and no controls and was located in the C-terminal region of the C4BP β-chain. Our results indicate a normal expression, secretion and polymerization of the β-chain T232A mutant.

In this study, four alterations found in CD46 were analyzed functionally. We found that the rare variant P324L, located in STP-C, caused significantly decreased expression level of CD46 on the surface of CHO cells as well as in cell lysates. N213I, located in CCP3, displayed normal cell surface expression level but the protein concentration in the cell lysate was decreased compared to WT. This might be explained by deficient processing of N213I to the mature form, as observed by western blotting. Retention of the precursor form in the endoplasmic reticulum is usually an indication of a misfolded protein. A similar phenomenon has previously been observed for several CD46 mutations found in aHUS patients [15, 20]. N213I also showed decreased cofactor activity for FI in the degradation of both C4b and C3b. We did not find any change in expression or cofactor activity for T383I, which is located in the intracellular Cyt-1 domain. However, it should be noted that this

change could disrupt a putative phosphorylation site for casein kinase-2 and protein kinase C [21]. Similarly, we did not detect any impairment for the P193S mutant, which interestingly lies in an indel. Proline 193 has previously been analyzed in a mutant carrying double mutation of proline 193 and aspartic acid 192 to alanines. This double mutant had slightly less cofactor activity compared to WT [22]. Interestingly, PolyPhen software predicted that some of the C4BP and CD46 alteration, which in our assays testing effect on complement showed normal function, could potentially be damaging. This may be related to yet unknown and thus untested functions of C4BP and CD46.

As shown previously the A353V polymorphism in CD46, a conservative amino acid substitution in the transmembrane domain, did not affect the ability of recombinant CD46 to bind C3b/C4b or to act as a cofactor to FI in the fluid phase. However, by an unclear mechanism the mutant was defective in its complement regulatory activity when embedded in the membrane [14]. In the present study, A353V was found in three patients and in 3.1% of 192 healthy controls of Caucasian origin. NCBI database (www.ncbi.nlm.nih.gov) estimates 2% frequency for this single nucleotide polymorphism (SNP) in European populations. These frequencies suggests that the A353V polymorphism in CD46 is not a strong causative factor for the diseases analyzed so far but could be a modifying factor in the presence of additional defects in complement regulation often observed in these patients.

One could argue that the currently studied phenotype, RSPL, is extremely vital from an evolutionary perspective. It is probably one of the most clearly evolutionarily driven phenotypes that can be identified and which is not necessarily lethal in itself. If a woman carries a specific genetic makeup, predisposing to pregnancy loss with high penetrance, she will simply not be able to pass these genes on. Thus, we can expect that this disorder could in some cases be dependent on highly penetrant rare variants rather than weakly predisposing common ones. Recent findings suggest that rare variants play an important role in both monogenic and common diseases. However, due to their rarity, it remains difficult to appropriately statistically analyze the association between such variants and disease. In this current study we used a relatively small cohort to analyze the occurrence of rare variants in three complement inhibitors. We further showed that several of these variants affect expression and/or function of the resulting proteins. However, to conclude if there is a significant association will require replication in larger cohorts in order to provide sufficient statistical power.

Taken together with previous results reported for preeclampsia, our exploratory study provides ground for further evaluation of rare variants in complement inhibitors in larger cohorts of patients characterized by pregnancy complications. Perhaps, it will parallel the findings obtained for aHUS, for which it is now well established on the basis of several independent cohorts that over 50% of patients with aHUS carry mutations in complement factors and inhibitors [23]. Indeed, these studies were inspired by pilot reports on small numbers of cases [15, 24, 25]. In conclusion, we report the functional consequences of several alterations found in C4BP and CD46 in patients with RSPL. These data will likely be useful in analysis of other disease cohorts in which similar alterations are identified.

## Materials and Methods

### Patients and controls

Patients were referred to the outpatient department of Gynecology and Obstetrics or the Hematology laboratory, University Hospital of Nîmes, France, from January 2002 to January 2004, for RSPL and relevant investigations. The 1359 women whose pregnancy loss had been objectively confirmed by transvaginal ultrasound exam were pre-selected. We focused on the 962 most severe primary cases, defined by at least three consecutive

embryonic losses before the 10th gestational week, or two consecutive fetal losses at and beyond the 10th gestational week, all occurring in childless women. A complete clinical summary with emphasis on personal history for thromboembolic disease and previous pregnancy losses was obtained from all subjects. Classical risk factors were excluded according to an invariable protocol developed by the involved medical staff. This included screens for abnormal parental karyotypes, infectious diseases during pregnancy (systematic HIV, hepatitis B/C and *Chlamydia trachomatis* serologies, toxoplasmosis serology), uterine anatomical abnormalities (hysterosalpingoscopy), diabetes mellitus, thyroid dysfunction, hyperprolactinaemia prior to luteal phase defects (a normal luteal phase of at least 12 days and plasma progesterone above 25 ng/ml), erythroblastosis fetalis (Rh disease), immune thrombocytopenic purpura, feto-maternal alloimmune thrombocytopenia, antiphospholipid antibodies performed according to available recommendations (lupus anticoagulant, anticardiolipin IgG and IgM antibodies, anti-β2GP1 IgG and IgM antibodies). Any data missing from this protocol led to the patient being excluded from the study (n=41). Any of these abnormalities identified led to the pregnancy loss being defined as favored by classical risk factors (n=327). Screening for constitutional thrombophilia was thereafter performed in the 594 remaining cases, including antithrombin, protein C and protein S (functional assays), fasting homocyteinemia, *F5* 6023 and *F2* rs1799963 polymorphisms. We finally excluded cases with: 1) any positive classical risk factor for pregnancy loss, 2) any previous occurrence of superficial or deep vein thrombosis, as a significant number of these women had already been investigated for thrombophilia prior to this study, leading to treatments during pregnancy interfering with the hemostatic system and possibly with the immune response (including heparins, low-dose or high-dose aspirin); 3) any constitutional thrombophilia; 4) preeclampsia, defined as gravidic hypertension (systolic blood pressure (BP) > 140 mm Hg, diastolic BP > 90 mm Hg, a rise in systolic BP > 30 mm Hg, or a rise in diastolic BP > 15 mm on at least two occasions, six hours apart) after 20 weeks and associated with a significant proteinuria (> 300 mg/24 h); 5) women of non-Caucasian grandparental origin, which may have introduced consistent confounding heterogeneities in the local frequencies of the polymorphisms and mutations under focus. A total of 453 women fulfilled all the criteria. After informed consent was obtained, 429 patients were finally recruited (327 cases with embryonic losses, 126 cases with fetal losses). Controls constituted DNA samples obtained from women referred to the outpatient department of Gynecology and Obstetrics for a systematic medical exam such as implementation of a new contraception or evaluation of the pelvic floor after pregnancy. We selected women with no previous pregnancy loss but at least two uneventful pregnancies (n=261). Similarly to cases, they were checked for classical risk factor for pregnancy loss (see protocol above), including antiphospholipid antibodies and constitutional thrombophilias, any positivity leading to exclusion. After informed consent was obtained, 224 controls were finally recruited. The study was approved by the University Hospital of Nîmes Institutional Review Board and ethics committee (Ref # 2001-12-07). This investigation was performed according to the Helsinki declaration.

## DNA sequencing

DNA sequencing using Sanger dideoxy method was performed by Polymorphic DNA Technologies (Alameda, CA). All exons, including at least 20 flanking intron nucleotides, were analyzed for *C4BPA, C4BPB, CD46* and *CD55*. *C4BPA, C4BPB, CD46* genes were analyzed in 384 randomly chosen patients from the cohort defined above while the *CD55* gene was sequenced in 192 patients. All exons containing mutations and polymorphisms identified in *C4BPA, C4BPB* and *CD46* were also sequenced in 192 randomly chosen controls. Frequencies of identified non-synonymous alterations in *C4BPA, C4BPB* and *CD46* were investigated in general populations using dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/) and NHLBI Exome Sequencing Project database

(http://evs.gs.washington.edu/EVS/). Potential effect of alterations on protein function was evaluated using PolyPhen software (http://genetics.bwh.harvard.edu/pph2/).

### Proteins

C3, factor B (FB), factor D (FD), C3b and C4b were purchased from Complement Technology. C3b and C4b were labeled with $^{125}$I, using the chloramine-T method [26]. The specific activity was 0.4–0.5 MBq/μg of protein. FI was purified from human plasma as described previously [27].

### Expression of *C4BPA* and *CD46* variants

For introduction of the found *C4BPA* alterations, cDNA coding for human C4BP α-chain in pBluescript (Stratagene) was used as template. The template for *CD46* was the isoform BC1 (GenBank accession no. X59405) in pSG5 (Stratagene) [28]. Site directed mutagenesis was performed, using the QuikChange site directed mutagenesis kit (Stratagene), according to manufacturer's instructions. Primers used for mutagenesis are presented in Table 4. The mutations were confirmed, using automated DNA sequencing. The constructs containing C4BP α-chain, WT and mutants, were cloned into the eukaryotic expression vector pcDNA3 (Invitrogen). Human embryonic kidney 293 (HEK 293) cells were transiently or stably transfected with C4BPα construct using Lipofectin (Invitrogen) and the protein purified using a mAb104 affinity column, as previously described [18]. The mutants were analyzed by SDS-PAGE, both under non-reducing (5% SDS-PAGE) and reducing (25 mM DTT, 10% SDS-PAGE) conditions, followed by Coomassie Brilliant Blue staining.

CD46 constructs were transiently transfected into CHO cells using Lipofectamine 2000 (Invitrogen). The cells were cultured in glutamine containing RMPI 1640 (Hyclone), supplemented with 10% FCS, 50 U/ml penicillin and 50 μg/ml streptomycin. The monoclonal TRA-2-10 antibody [29] (a generous gift from Prof. Andrews, Sheffield University), directed against CCP1, where no mutations analyzed were situated, was used for detection of the cell surface expression of CD46 by flow cytometry (Partec), as described [30]. Cell lysates were also subjected to western blotting, after transient transfection. Cells were lysed ($2\times10^7$ cells/ml) by adding 1% Nonidet P-40, 0.05% SDS, 2 mM PMSF in TBS for 15 min at 4°C, followed by centrifugation at 12,000 × g for 10 min. The supernatant from an equivalent of 200,000 cells were analyzed under reducing conditions (25 mM DTT) on a 10% SDS-PAGE gel, transferred to a polyvinylidene fluoride membrane and CD46 was then detected using TRA-2-10. Detection of β-actin (Abcam) was used a loading control. For determination of the CD46 concentration in cell lysates after transfections, an ELISA assay was performed as described [31]. The two antibodies used in the ELISA recognize both the mature and precursor form of CD46 [20].

### Simultaneous expression of *C4BPA* and *C4BPB*

The pBudCE4.1 vector (Invitrogen) was used for simultaneous expression of the two genes *C4BPA* and *C4BPB*. *C4BPB* was cloned into the CMV multiple cloning site, using HindIII and PstI restriction enzyme sites, followed by introduction of the T232A mutation using the QuikChange mutagenesis kit. Primers used for mutagenesis are presented in Table 1. *C4BPA* was subsequently cloned into the EF-1α multiple cloning site, using XhoI and NotI restriction enzyme sites. After transient transfection of HEK 293 cells with pBudCE4.1 using lipofectamine 2000, the cell medium was collected and the cells lysed, as described above. The amount of both α-chain containing C4BP (*i.e.* total C4BP) and β-chain containing C4BP, were detected using ELISA, as described [32].

### The cofactor activity of C4BP for C4b and C3b degradation in fluid phase

To elucidate whether the cofactor activity of C4BP to FI, in the degradation of C3b or C4b, was affected in fluid phase, a degradation assay was performed, essentially as described [19]. C4BP, at a concentration of 0–80 μg/ml, was mixed in TBS together with 50 μg/ml C4b (or 150 μg/ml C3b), 8 μg/ml FI and trace amounts of $^{125}$I-labeled C4b (or $^{125}$I-C3b), in a total volume of 40 μl. The samples were incubated for 1.5 h at 37°C and the reaction was terminated by the addition of SDS-PAGE sample buffer, containing a reducing agent (25 mM DTT). The samples were incubated at 95°C for 3 min and applied on a 10–15% gradient SDS-PAGE. The radioactive gels were visualized by autoradiography, using Fluorescent Image Analyzer, FLA-3000 (FujiFilm, Japan) and the intensity of the bands were analyzed with ImageGauge software (FujiFilm). Data are shown as the ratio of the intensity of the band corresponding to intact α'-chain of C4b or C3b and the C4b degradation product C4d or the 43 kDa degradation product of C3b.

### The cofactor activity of C4BP to C3b degradation on the cell surface

C4BP also acts as a cofactor to FI in the degradation of C3b deposited on a surface and this function was tested essentially as described previously [19]. In this assay, sheep erythrocytes were coated with C3b and incubated for 30 min at 37°C together with 5 μg/ml FI and 0–200 μg/ml C4BP. To control the amount of deposited C3b on the erythrocytes as well as generated degradation product iC3b, the cells were incubated with mouse monoclonal antibodies directed against human C3d (which recognizes C3b) or iC3b antibody (3 μg/ml of each antibody, Quidel) in PBS, supplemented with 1% BSA (Saveen Werner AB), 15 mM EDTA and 30 mM $NaH_3$. Matched FITC-labeled secondary antibodies (10 μg/ml, Dako) were added and the samples analyzed by flow cytometry (Partec).

### Binding of CD46 to C4b and C3b

To evaluate the capacity of the CD46 variants to bind C4b and C3b, a microtiter plate binding assay was performed, as described [31]. As negative controls, corresponding volumes of cell lysate from mock-transfected (empty pSG5 vector) CHO cells were analyzed.

### The cofactor activity of CD46 for C4b and C3b degradation

To determine whether the FI-mediated cofactor activity of CD46 for C4b and C3b was affected by the mutations, degradation assays were performed. Cell lysates were diluted to a CD46 concentration of 10 nM (for C4b) or 2 nM (for C3b) in 10 mM Tris (pH 7.4), 25 mM NaCl, together with 50 μg/ml C4b (or 150 μg/ml C3b), 8 μg/ml FI and trace amounts of $^{125}$I-labeled C4b (or $^{125}$I-C3b). The total reaction volume was 40 μl. As negative controls, corresponding volumes of cell lysate from mock-transfected (empty pSG5 vector) CHO cells were analyzed. The samples were incubated at 37°C for 30, 60 and 90 min and the reaction was terminated and analyzed as described above for C4BP.

### Statistical analyses

All experiments were done at least three independent times. The results are shown as means ± SD. Statistical significance was determined using either Fisher's exact test, one-way ANOVA with Dunnett's multiple comparison test or two-way ANOVA with Bonferroni's post-test. Values of $p < 0.05$ were considered significant (*$p < 0.05$, **$p < 0.01$; and ***$p < 0.001$).

## Acknowledgments

The authors would like to acknowledge helpful advice of Dr. Claes Ladenvall (Lund University) and Michael Triebwasser (Washington University School of Medicine) and the financial support of the Söderberg Foundation, the Swedish Research Council (K2012-66X-14928-09-5), the National Board of Health and Welfare and Skåne University Hospital as well as the Foundations of Österlund, Greta and Johan Kock, Knut and Alice Wallenberg and Inga-Britt and Arne Lundberg. This work was also supported by National Institutes of Health R01 GM099111-17 (J.P.A.).

## Abbreviations

| | |
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| **aHUS** | atypical hemolytic uremic syndrome |
| **BP** | blood pressure |
| **CCP** | complement control protein domain |
| **Cyt-1/2** | cytoplasmic tail 1/2 |
| **C4BP** | C4b-binding protein |
| **DAF** | decay accelerating factor (CD55) |
| **FB** | factor B |
| **FD** | factor D |
| **FI** | factor I |
| **HEK** | human embryonic kidney |
| **MCP** | membrane cofactor protein (CD46) |
| **RSPL** | recurrent spontaneous pregnancy loss |
| **SLE** | systemic lupus erythematosus |
| **SNP** | single nucleotide polymorphism |
| **STP** | serine, threonine and proline region |

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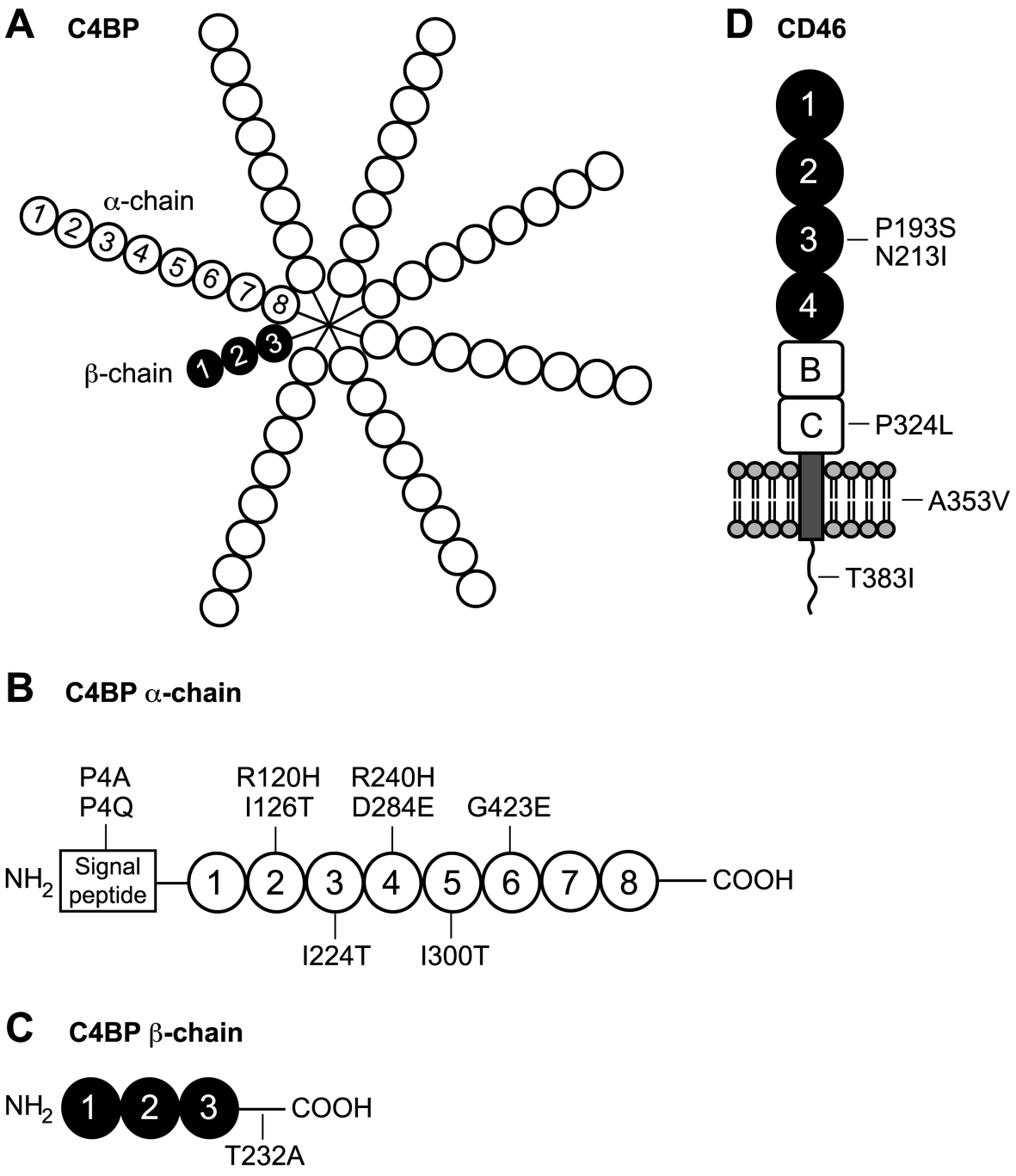


**Figure 1. Schematic representation of C4BP and CD46 with indicated mutations and polymorphisms identified in this study**

(A) The major form of C4BP is composed of seven identical α-chains and one unique β-chain. The chains are covalently linked by their C-termini and form a spider-like structure. The α- and β-chains contain eight and three CCP domains respectively. (B) The C4BP α-chain, together with the signal peptide, which is cleaved off from the mature protein, is shown. The nine coding alterations identified in this study are indicated. (C) The location of the mutation found in the C-terminus of C4BP β-chain. (D) CD46 is composed of four complement control protein (CCP) domains; serine, threonine and proline rich domain/s (STP), one transmembrane domain and finally a cytoplasmic tail. Alternative splicing of the

STP region results in A, B or C variants, of which BC is the most common. The cytoplasmic tail can also be alternatively spliced and give rise to either cytoplasmic tail 1 or 2. Coding alterations found in this study are indicated in the figure and since we found one mutation in STP-C and one in cytoplasmic tail 1, we used the BC1 construct in this study.

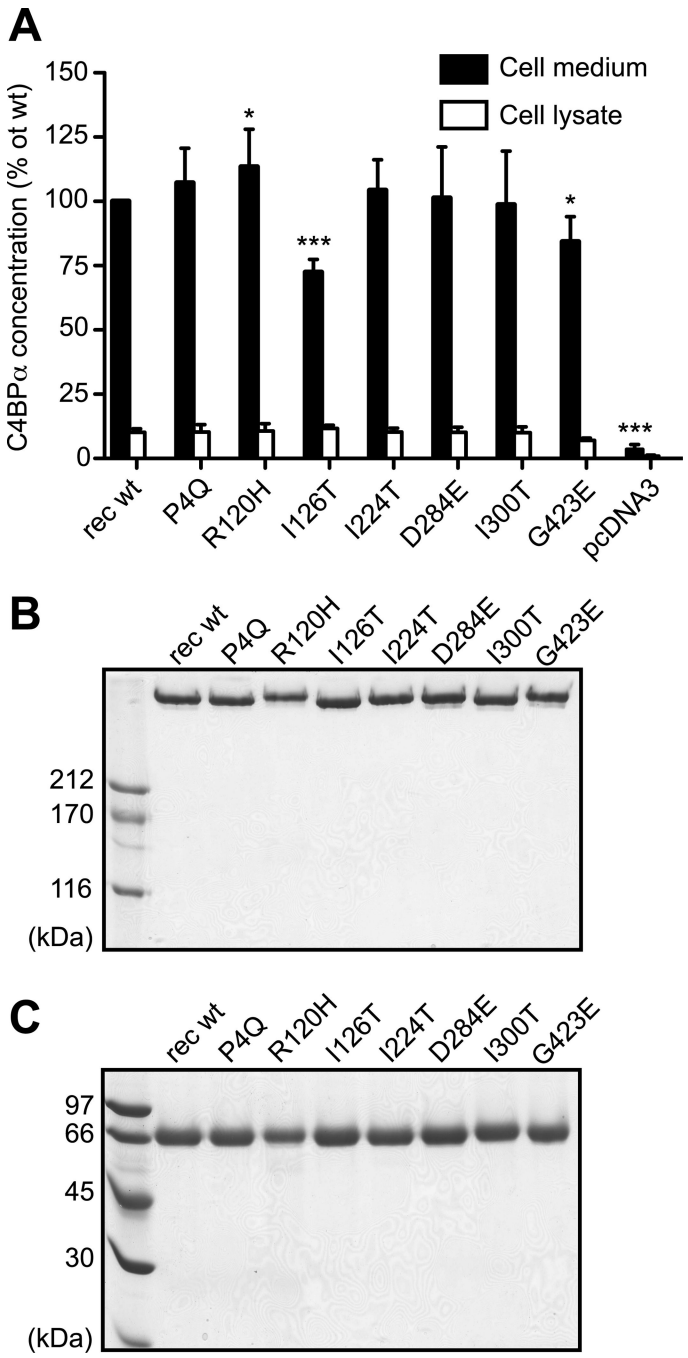


**Figure 2. Expression of C4BP α-chain variants**

(A) C4BP α-chain WT and the variants (or empty pcDNA3 vector as a negative control) were transiently transfected into HEK 293 cells and the C4BP concentration in conditioned cell media and cell lysates was determined using ELISA. Each sample was analyzed in duplicates and the results are shown as mean + SD of three independent transfection experiments. Statistical significance of the differences between WT and mutants were evaluated using two-way ANOVA with Bonferroni's post-test, *$p<0.05$, ***$p<0.001$. (B–C) After stable expression and purification of C4BP α-chain WT and the variants, the proteins were subjected to SDS-PAGE, followed by Coomassie Brilliant Blue staining under both

(B) non-reducing and (C) reducing conditions. Data shown are representative of two experiments.

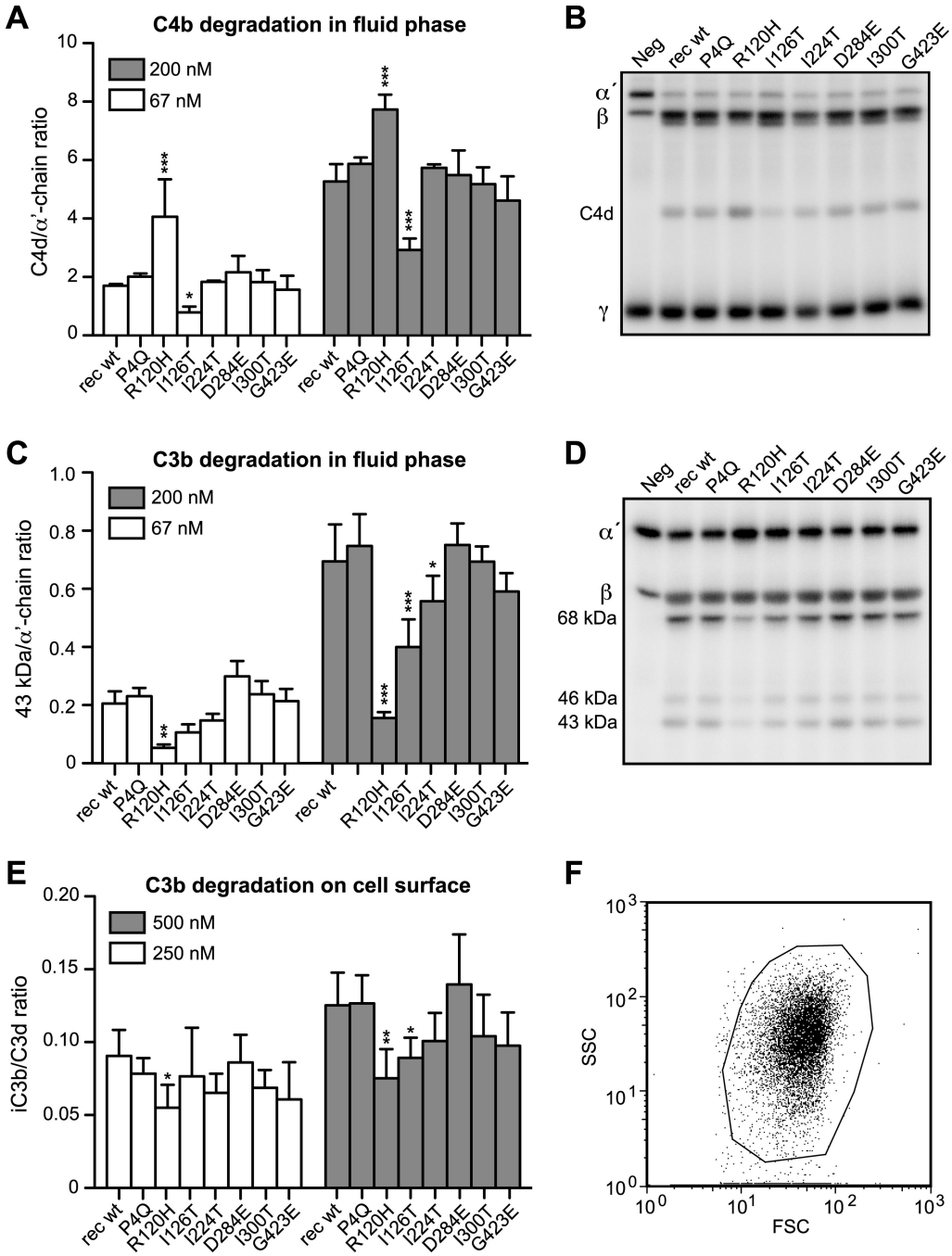


**Figure 3. Functional activity of C4BP WT and the variants**

C4BP WT and the variants were expressed in a stable manner, purified and their cofactor function to FI tested in the degradation of (A, B) C4b in the fluid phase, (C, D) C3b in the fluid phase or (E, F) C3b on the surface of erythrocytes. (A) The band corresponding to intact α'-chain of C4b as well as the degradation product C4d were quantified by densitometry. Data are shown as ratio of the intensity of the two bands. (B) A representative gel of C4b degradation is shown. (C) The band corresponding to intact α'-chain of C3b as well as the 43 kDa degradation product were quantified by densitometry. Data are shown as ratio of the intensity of the two bands. (D) A representative gel of C3b degradation is shown. (E) The amounts of the degradation product iC3b and C3d (antibody also recognizes C3b)

on the surface of erythrocytes were detected by flow cytometry and the data are shown as the ratio of these two signals, (F) in the gated cell population. (A, C, E) Each sample was analyzed as a single replicate and data are shown as mean + SD from at least three independent experiments. Statistical significance of the differences between WT and mutants were evaluated using two-way ANOVA with Bonferroni's post-test, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

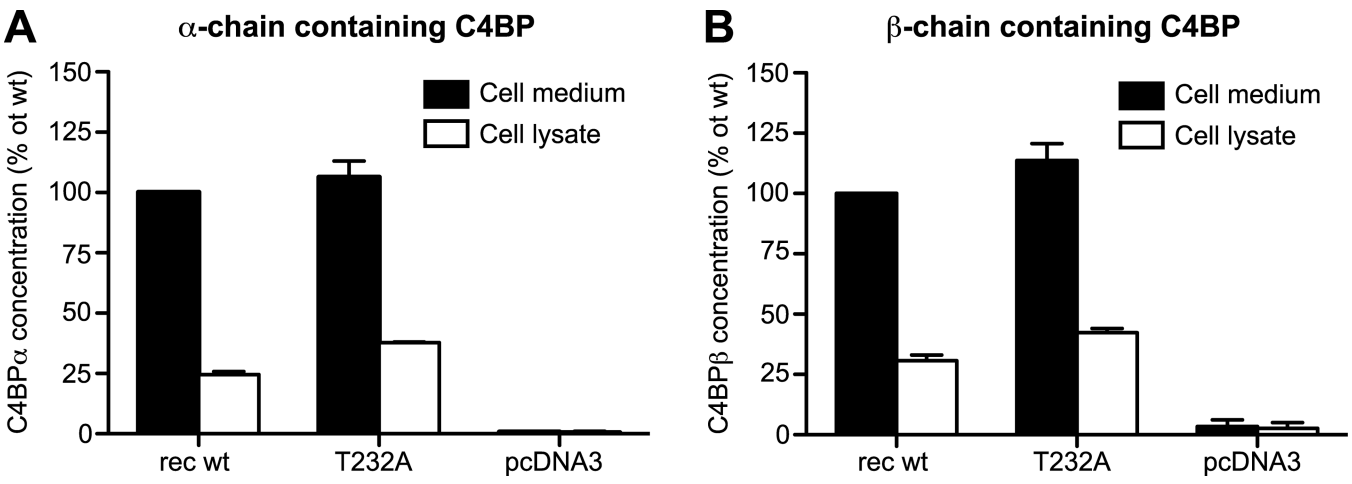


**Figure 4. Simultaneous C4BP α- and β-chain expression**

Bicistronic vector was used to express simultaneously the α- and β-chain of C4BP, the latter one either in WT form or carrying the T232A mutation. Empty pcDNA3 vector was used as a negative control. The concentration of C4BP in cell media and cell lysates were then determined using ELISA, detecting (A) the α-chain or (B) the β-chain. Each sample was analyzed in duplicate and the results are shown as mean + SD of three independent transfection experiments. No statistical significant difference between WT and mutant was found using two-way ANOVA with Bonferroni's post-test.

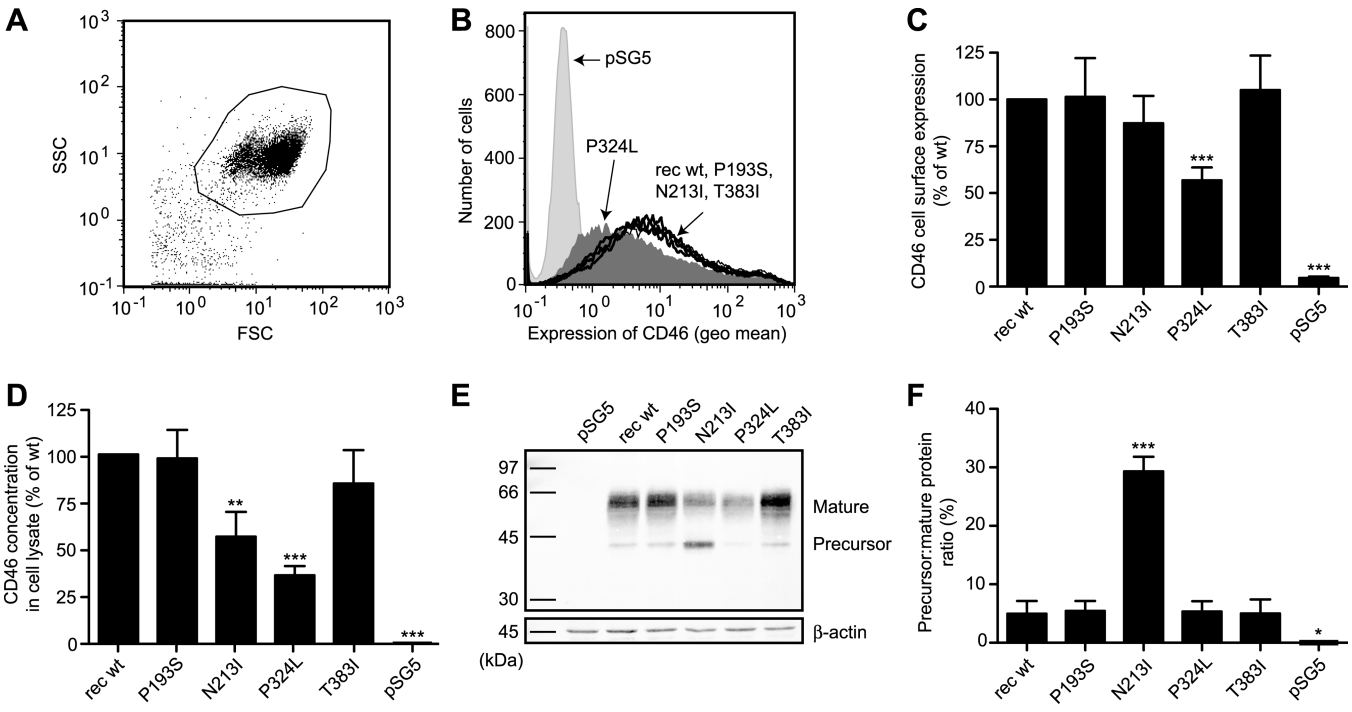


**Figure 5. Expression of CD46 WT and variants**

CD46 WT and the identified variants (or empty pSG5 vector as a negative control) were transfected into CHO cells. (A–C) The CD46 expression on the cell surface was determined using flow cytometry. (A) A representative dot plot showing gating of the cells. (B) A representative histogram showing flow cytometric determination of the CD46 expression and (C) the geometric mean was calculated. (D) The CD46 concentration was determined in cell lysates using ELISA. (E, F) Cell lysates were also analyzed with western blotting. (E) The size of the mature and precursor CD46 is approximately 60 kDa and 40 kDa respectively. β-actin was used as a loading control. (F) The ratio between precursor and mature form of CD46 were quantified using densitometry. (C, D, F) Each sample was analyzed in single or duplicate and the data are shown as mean + SD from at least three independent transfection experiments. Statistical significance of the differences between WT and mutants was evaluated using one-way ANOVA with Dunnett's multiple comparison test, *p<0.05, **p<0.01, ***p<0.001.

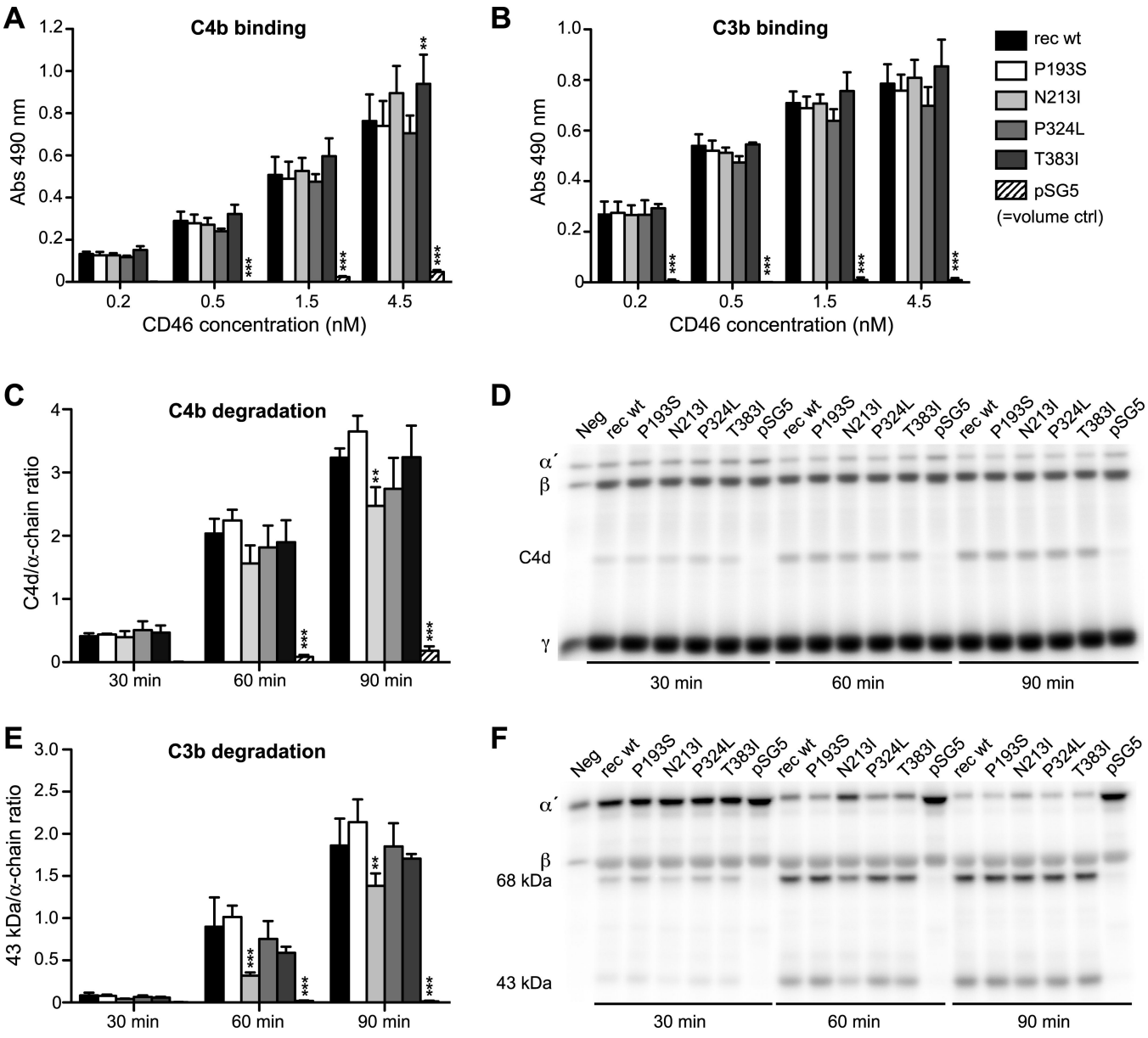


**Figure 6. Functional activity of CD46 WT and variants**

CD46 WT and variants were transfected into CHO cells. Cell lysates were prepared and the CD46 concentration in these were determined using ELISA. Cells transfected with empty pSG5 vector did not express any CD46 (as shown in Fig. 5D) and corresponding volumes of this cell lysate were used as a negative control. (A, B) The ability of the CD46 variants to bind to (A) C4b and (B) C3b were analyzed in a microtiter plate assay. (C–F) The FI cofactor activity of CD46 from the cell lysates, in the degradation of (C, D) C4b and (E, F) C3b, was determined after transfection. Representative gels for (D) C4b degradation and (F) C3b degradation are shown. The bands corresponding to intact α´-chain as well as the C4b degradation product C4d and the 43 kDa degradation product of C3b were quantified by densitometry. (C, E) Results are depicted as the ratio of the intensity of the α´-chain and the product. Each sample was analyzed in single or duplicate and the data are shown as mean + SD from at least three independent transfection experiments. Statistical significance of the

differences between WT and mutants was evaluated using two-way ANOVA with Bonferroni's post-test, *$p<0.05$, **$p<0.01$.

**Table 1**

Mutations and polymorphisms found in *C4BPA* and *C4BPB*[a)].

| Amino acid[b)] | cDNA ATG +1 | SNP number | Exon | Domain | Polyphen[c)] | MAF, % (dbSNP/NHLBI)[d)] | Frequency in patients, % (no.) | Frequency in controls, % (no.) |
|---|---|---|---|---|---|---|---|---|
| ***C4BPA*** | | | | | | | | |
| P4Q | c.11 C>A | rs55867570 | 2 | Signal sequence | Benign | 2.7/3.6 | 5.47 (21) | 6.77 (13) |
| P4A | c.10 C>G | rs12072216 | 2 | Signal sequence | Benign | 1.5/0.03 | 0.52 (2) | 0.52 (1) |
| R120H | c.359 G>A | novel | 4 | CCP2 | Benign | 0/0 | 0.52 (2) | 0 (0) |
| I126T | c.377 T>C | novel | 4 | CCP2 | Possibly damaging | 0/0 | 0.26 (1) | 0 (0) |
| I224T | c.671 T>C | rs116795518 | 6 | CCP3 | Benign | 1.1/0.03 | 0.52 (2) | 0.52 (1) |
| R240H | c.719 G>A | rs45574833 | 7 | CCP4 | Benign | 0.6/1.3 | 1.82 (7) | 2.08 (4) |
| D284E | c.852 T>G | novel | 4 | CCP4 | Possibly damaging | 0/0 | 0.26 (1) | 0 (0) |
| I300T | c.899 T>C | rs4844573 | 8 | CCP5 | Benign | 46.8/34.7 | 40.1:47.9:11.7[e)] (154:184:45) | 42.7:43.2:13.8[e)] (82:83:26) |
| G423E | c.1268 G>A | rs116700161 | 9 | CCP6 | Benign | 0.1/0.5 | 0.26 (1) | 1.04 (2) |
| ***C4BPB*** | | | | | | | | |
| T232A | c.694 A>G | rs141922788 | 6 | C-terminus | Possibly damaging | NA[f)]/0.02 | 0.26 (1) | 0 (0) |

[a)] The mutations were found in heterozygous form. None of the alterations were significantly associated with the disorder, as determined by Fisher's exact test.

[b)] Numbering including signal peptide, Met = 1

[c)] PolyPhen prediction of effect on protein function according to http://genetics.bwh.harvard.edu/pph2/

[d)] MAF, minor allele frequency in dbSNP database and NHLBI Exome Sequencing Project database.

[e)] TT:TC:CC

[f)] NA, not available

**Table 2**

Mutations and non-synonymous polymorphisms found in *CD46*[a)].

| Amino acid[b)] | cDNA ATG +1 | SNP number | Exon | Domain | PolyPhen[c)] | MAF, % (dbSNP/NHLBI)[d)] | Frequency in patients, % (no.) | Frequency in controls, % (no.) |
|---|---|---|---|---|---|---|---|---|
| P193S | c.577 C>T | unknown | 5 | CCP3 | Probably damaging | 0/0.01 | 0.26 (1) | 0 (0) |
| N213I | c.638 A>T | novel | 5 | CCP3 | Probably damaging | 0/0 | 0.26 (1) | 0 (0) |
| P324L | c.971 C>T | rs41317833 | 9 | STP-rich region C | Probably damaging | 0.09/0 | 0.26 (1) | 0 (0) |
| A353V | c.1058 C>T | rs35366573 | 11 | transmembrane | Benign | 0.8/1.7 | 1.04 (4) | 3.1 (6) |
| T383I | c.1148 C>T | rs146803767 | 13 | cytoplasmic tail 1 | Benign | 0.05/0.06 | 0.26 (1) | 0 (0) |

a)The mutations were found in heterozygous form. None of the identified alterations were significantly associated with the disorder, as determined by Fisher's exact test.

b)Numbering including signal peptide, Met = 1; longest isoform ABC with Cyt-1

c)PolyPhen prediction of effect on protein function according to http://genetics.bwh.harvard.edu/pph2/

d)MAF, minor allele frequency in dbSNP database and NHLBI Exome Sequencing Project database.

## Table 3

Description of patients carrying heterozygous *CD46, C4BPA* and *C4BPB* alterations, which alter expression or function.

| Patient code | Age at recruitment | Pregnancy loss | Medical history | Alterations found |
|---|---|---|---|---|
| A742 | 38 years | 3 spontaneous miscarriages at 5, 6 and 5 weeks of gestation, one fetal loss at 12 weeks | Personal antecedents: atopia (asthma, chronic eczema). Normal clinical exam. First-degree relatives: insulin-dependent diabetes mellitus in the mother, allergic sinusitis in the brother. | *C4BPA*: G423E *C4BPB*: T232A |
| A706 | 32 years | 5 spontaneous miscarriages at 6, 7, 5, 6 and 5 weeks of gestation. One fetal loss at 12 weeks, as she was empirically taking a low-dose aspirin treatment. | No previous medical problems. Clinical exam: limited venous angiomatosis on one lower limb, two hepatic hemangiomas on CT scan. First degree relatives: 3 spontaneous miscarriages in the mother, then a small-for-gestational age premature infant birth under progesterone supplementation, 32 weeks of gestation. | *C4BPA*: R120H |
| A975 | 38 years | 3 spontaneous miscarriages at 8, 4 and 9 weeks of gestation | No previous medical problems. Normal medical exam. First-degree relatives: chronic coronary insufficiency in the father; asymptomatic brother and young sister (22 years old, no pregnancy attempt). | *CD46*: N213I |
| A1001 | 29 years | 3 spontaneous miscarriages at 5, 6 and 5 weeks of gestation. | No previous medical problems. Clinical exam: chronic venous insufficiency, telangiectasias. First degree relatives: one unexplained late, third trimester fetal loss in the mother, then a spontaneous small-for-gestational age premature infant birth, 35 weeks of gestation. | *C4BPA*:I126T |
| A1024 | 30 years | 4 spontaneous miscarriages at 7, 6, 5 and 5 weeks of gestation | No previous medical problems. Normal clinical exam. First degree relatives: allergic sinusitis in the mother, premature chronic coronary and cerebrovascular insufficiency in the father. | *C4BPA*: R120H |
| A1144 | 30 years | 2 spontaneous fetal losses, each at 10 weeks of gestation | No previous medical problem. Medical exam: suspicion of dysplastic nevi syndrome, confirmed further on by dermatologists. No identified illness in first-degree relatives, including two sisters, each of them with a normal pregnancy. | *C4BPA*: P4Q *CD46*: P324L |

## Table 4

Primer sequences (5´to 3´) used to introduce site directed mutations.

| Gene | Mutation | Primer sequence (5' to 3')[a)] |
|---|---|---|
| *C4BPA* | P4Q | CAGGCCATGCACCCCCAAAAAAACTCCATCTGGG |
| | R120H | CCCAGGAGAGTTACATAATGGGCAAGTAGAG |
| | I126T | GGGCAAGTAGAGACTAAGACAGATTTATC |
| | I224T | GTGGAGAATGAAACAACAGGTGTTTGGAGACCA |
| | D284E | CATTGTGATGCTGAGAGCAAATGGAATCC |
| | I300T | CCCAATAGTTGTACTAATTTACCAGAC |
| | G423E | CACCATCATGTGAAGACATTTGCAATTT |
| *C4BPB* | T232A | GGAAAGTGGCATGGCAATGGAGGAGC |
| *CD46* | P193S | CTTATAGTTGTGATTCTGCACCTGGACCAG |
| | N213I | CGATTTATTGTGGTGACATTTCAGTGTGGAG |
| | P324L | CCTACTTACAAGCCTCTAGTCTCAAATTATCC |
| | T383I | CCTAACTGATGAGATCCACAGAGAAG |

a)Nucleotides corresponding to the changed amino acid residue are underlined.