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Presenilin/γ-Secretase Cleaves CD46 in Response to *Neisseria* Infection

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Abstract

CD46 is a type I transmembrane protein with complement and T cell regulatory functions in human cells. CD46 has signaling and receptor properties in immune and nonimmune cells, many of which are dependent on the expression of cytoplasmic tail (cyt) isoforms cyt1 or cyt2. Little is known about how cyt1 and cyt2 mediate cellular responses. We show that CD46-cyt1 and CD46-cyt2 are substrates for presenilin/γ-secretase (PS/γS), an endogenous protease complex that regulates many important signaling proteins through proteolytic processing. PS/γS processing of CD46 releases immunoprecipitable cyt1 and cyt2 tail peptides into the cell, is blocked by chemical inhibitors, and is prevented in dominant negative presenilin mutant cell lines. Two human pathogens, *Neisseria gonorrhoeae* and *Neisseria meningitidis*, stimulate PS/γS processing of CD46-cyt1 and CD46-cyt2. This stimulation requires type IV pili and PilT, the type IV pilus retraction motor, implying that mechanotransduction plays a role in this event. We present a model for PS/γS processing of CD46 that provides a mechanism by which signals are transduced via the cyt1 and cyt2 tails to regulate CD46-dependent cellular responses. Our findings have broad implications for understanding the full range of CD46 functions in infection and noninfection situations.

The immunomodulatory protein, CD46, is best recognized for its role in regulating the complement cascade (1). CD46 also participates in adaptive immunity by acting as a costimulatory molecule that promotes T cell proliferation (2, 3) and differentiation of T regulatory type 1 (Tr1) cells (4). CD46/CD3 costimulation drives differentiation of $CD4^+$ lymphocytes into Tr1 cells and promotes production of the immunosuppressive cytokine IL-10 and cytotoxic granzyme B (4, 5). Ab ligation of CD46 affects cytotoxic T cell function by altering its ability to interface with APCs (6). Ab ligation of CD46 in immune cells

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triggers tyrosine phosphorylation by src kinase Lck (7); phosphorylation of the adaptors $p120^{CBL}$ and linker for activation of T cells; and activation of MAPK ERK, guanine nucleotide exchange factor Vav, and GTPase Rac (2, 8). The most abundant CD46 isoforms contain one of two short cytoplasmic tails (cyt), cyt1 or cyt2. Both tails share putative signaling and nuclear localization motifs (7, 9) and a number of tail-specific signaling functions have been identified (10–12). Despite the numerous activities described for CD46, little is known about the mechanism(s) it uses to propagate signals.

Several pathogens elicit cellular responses through CD46. Measles virus and human herpesvirus 6 inhibit production of the proinflammatory cytokine IL-12 in primary human monocytes and human macrophages, respectively (13, 14). A similar inhibition of IL-12 production occurs on Ab ligation of CD46 (13). CD46-group A streptococcal M protein interactions promote differentiation of Tr1-like cells, IL-10 secretion, and granzyme B production (15). Pathogens may thus induce CD46 signaling pathways to influence immune cell function (5, 13, 16).

Several pathogens induce the downregulation of CD46 (17–21), a response that also occurs on Ab ligation of CD46 on nonlymphoid cells (22). Adenovirus, measles virus, and human herpesvirus 6 infection lower CD46 levels at the infected cell surface (20, 21, 23). Group A streptococcal M protein induces surface downregulation and extracellular shedding of CD46 (19).

Neisseria gonorrhoeae and *Neisseria meningitidis* interact with CD46 at multiple levels. *Neisseria* type IV pili (Tfp) are retractile fibers that promote neisserial adhesion and epithelial cell signaling (24, 25). Tfp fibers bind to CD46 invitro (26). Tfp-mediated infection by *N. gonorrhoeae* induces cyt2 tyrosine phosphorylation by src kinase c-Yes (27). In Jurkat cells, the same residue is phosphorylated by src kinase Lck on Ab ligation of CD46 (7). Finally, Tfp-mediated *N. gonorrhoeae* infection induces the downregulation of surface and intracellular CD46 (18) by stimulating infected cells to shed $CD46^{+}$ vesicles (28). Tfp-induced downregulation of CD46 is dependent on PilT, the Tfp retraction motor (28).

N. gonorrhoeae recruits high concentrations of CD46 to the host cell cortex directly beneath adherent bacteria in a Tfp-dependent manner (28, 29). More than 99% of cortical plaques react with the CD46 ectodomain mAb; however, only a fraction of the plaques react with cyt1 or cyt2 tail-specific mAbs (29). This observation led us to question whether the CD46 ectodomain in the plaque is connected to the cytoplasmic tail. We tested the hypothesis that *N. gonorrhoeae* stimulates proteolytic cleavage of CD46 during infection. As CD46 is a type I transmembrane protein, we focused on presenilin/γ-secretase (PS/γS), a membrane protease complex that influences type I transmembrane protein function and signaling through proteolytic processing. More than 50 PS/γS substrates have been identified (30–32); they function in development [Notch (33)], cell adhesion [CD44 (34)], cancer [ErbB4 (35)], and the pathogenesis of Alzheimer's disease [amyloid precursor protein (APP) (36)]. Presenilin is the catalytic subunit of the γ-secretase complex [reviewed in (37)]. The two major presenilin isoforms, PS1 and PS2, have overlapping tissue distribution and substrate specificity (38, 39). Generally, for PS/γS processing to occur, the ectodomain of substrates must first be cleaved by extracellular sheddases, such as matrix metalloproteinases (MMPs).

MMPs cleave the protein near the plasma membrane, generating a soluble ectodomain and a membrane-anchored C-terminal fragment (40, 41). PS/γS then cleaves the C-terminal fragment within the transmembrane segment to generate a cytoplasmic intracellular domain (ICD). Several studies have shown that MMPs cleave CD46 (42–44); however, the role of PS/γS in CD46 processing has not been examined.

We determined whether *Neisseria* stimulates PS/γS to process CD46. We present the first evidence that the CD46-cyt1 and CD46-cyt2 isoforms are substrates of PS/γS. The role of PS/γS in CD46 processing was demonstrated using chemical inhibitors and dominant negative (DN) presenilin mutant cell lines. Low levels of cyt1 and cyt2 tail peptides are present in uninfected cells. *Neisseria* infection causes these levels to increase in a Tfp- and *pilT*-dependent manner. We propose a model for PS/γS processing of CD46 that describes a mechanism for how cyt1 and cyt2 tail peptides act as signaling intermediates. These studies will shed light on the pathways by which CD46 mediates its cellular responses, and by which pathogens regulate these processes.

Materials and Methods

Reagents and cell lines

PS/γS inhibitor L-685,458, DAPT (N-[N-(3,5-difluorophenacetyl-L-alanyl)]-S-phenylglycine *t*-butyl ester), GM6001, and the negative analog of GM6001 were purchased from Calbiochem (EMD Chemicals, Darmstadt, Germany), and Compound E from AXXORA (San Diego, CA). Anti–β-tubulin E7 mAb was purchased from Developmental Studies Hybridoma Bank (Iowa City, IA), anti-GAPDH rabbit mAb from Cell Signaling Technology (Danvers, MA), and anti–c-myc clone 9E10 from Sigma-Aldrich (St. Louis, MO). DMSO and isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from Sigma-Aldrich.

End1, a human endocervical cell line (45), was maintained in EpiLife (Invitrogen, San Diego, CA) supplemented with a human keratinocyte growth supplement (0.2% v/v bovine pituitary extract, 5 μg/ml bovine insulin, 0.18 μg/ml hydrocortisone, 5 μg/ml bovine transferrin, 0.2 ng/ml human epidermal growth factor), and 0.4 mM calcium chloride. Chinese hamster ovary (CHO) cell lines (kind gift from J. Atkinson) were maintained in Ham's F12 medium supplemented with 10% FBS, 4 mM L-glutamine, and 0.4 mg/ml G418. 16HBE14o- bronchial epithelial cells (46) were maintained in Eagle's MEM, 10% FBS with 2 mM L-glutamine, and grown in tissue culture vessels coated with a solution of LHC (Laboratory of Human Carcinogenesis) basal medium (Invitrogen) supplemented with 0.01 mg/ml human fibronectin (BD Laboratories, New York City, NY), 0.029 mg/ml bovine collagen (BD Laboratories), and 0.1 mg/ml BSA (Invitrogen). Prior to infection, 16HBE14o- cells were serum starved for 2 h to overnight in MEM and 2 mM L-glutamine.

Construction of DN cell lines

16HBE14o- cells were transfected with a plasmid encoding PS1-D385E, a mutation in presenilin with a DN phenotype, or the empty vector (pAG3hyg) (47). Hygromycin-resistant colonies were isolated using standard dilution cloning techniques. PS1-D385E-expressing

clones were identified by screening for APP C-terminal fragment accumulation, an indicator of reduced PS/γS activity, using an anti-APP polyclonal Ab (Abcam, ab12270). Clones with higher levels of APP C-terminal fragments were transfected with plasmid PS2-D366A (48). Stable double DN PS1/PS2-expressing cells were generated by selection for G418- and hygromycin-resistant clones. A control cell line stably expressing both empty parent vectors (pAG3hyg and pCDNA3.1) was also isolated in a similar manner. Presenilinase activity of presenilin was monitored to identify DN PS1/PS2-expressing clones (48).

γ-Secretase activity reporter assays

16HBE14o- cells were grown to 70% confluence in 35-mm dishes and transfected with Notch ΔE using FuGENE HD Transfection Reagent (Roche, Basel, Switzerland). Notch ΔE is an N-terminally truncated form of Notch that is a ligand-independent substrate of PS/γS and has a 6-myc tag at its C terminus (49, 50). Cleavage of Notch ΔE results in release of a ~60 kDa Notch ICD. At 24 h posttransfection, the cultures were harvested into cell lysis buffer and resolved on 7.5% Criterion SDS-PAGE gels (Bio-Rad, Hercules, CA) and probed with anti–c-Myc mAb 9E10. Levels of the Notch ICD served as a reporter for the level of PS/γS activity (48, 51).

Bacterial strains and infections

The *N. gonorrhoeae* strains used for this study are derivatives of strain MS11 (52). MS11-307 (Δ*pilE1::Erm* Δ*pilE2*) is mutated in pilin expression loci *pilE1* and *pilE2*; it produces no Tfp and does not adhere to cells (53, 54). MS11*pilT* is a *pilT*-null mutant that produces nonretractable Tfp, is nonmotile but adheres to cells; MS11*pilTi* has an IPTG-inducible *pilT* (25, 55, 56). *N. meningitidis* strain 8013 is a capsulated serogroup C isolate that produces highly adhesive Tfp (57). Bacteria were grown on GCB agar (Difco, Becton Dickinson, Franklin Lakes, NJ) with Kellogg's supplements I and II at 37°C in the presence of 5% CO_2. Piliated, Opa non-expressing strains were used for infections. Piliation and Opa status of bacteria were monitored by microscopic observation of colony morphology and immunoblotting with mAbs SM1 and 4B12 against pilin and Opa, respectively (58, 59). *pilT* expression was induced with 4 mM IPTG in GCB agar and in tissue culture medium. Mock infections were carried out using the appropriate medium. For the PS/γS inhibitor experiments, the vehicle control was DMSO. For infections, cells were grown to 95% confluence in 10-cm dishes unless otherwise stated.

At time of harvest, cells were washed three times with cold PBS and scraped into ice-cold lysis buffer consisting of 1% Triton X-100 (w/v), 1% deoxycholate, 0.1% SDS, 0.15 M NaCl, 50 mM Tris-Cl pH 7.2, 5 mM EDTA, 1 mM sodium orthovanadate, 50 mM NaF, 5mM 1,10-phenanthroline monohydrate and a 1× protease inhibitor mixture (Complete, EDTA-free, Roche).

Immunoprecipitations, immunoblotting, and quantitation of CD46 fragments

All samples were processed at 4°C. Nuclei and insoluble cell debris were removed from cellular lysates by centrifugation at 13,200×*g* for 30 min. Lysates were precleared for 30 min with 1 μg normal mouse IgG and Protein G Plus agarose (Santa Cruz Biotechnology, Santa Cruz, CA). Cell lysates were incubated with 5 μg mouse mAb 2F1 or mAb 13G10 directed

against the CD46 cyt1 and cyt2 tail, respectively (29). After a 1-h incubation, Protein G Plus agarose beads were added and incubated overnight. The beads were washed three times with PBS containing phosphatase and protease inhibitors (previously described), then suspended in 20 μl loading buffer (200 mM Tris-HCl pH 6.8, 40% glycerol, 2% SDS, 284 mM 2-ME), and heated for 5 min at 100°C. Proteins were separated by 10–20% Tris-Tricine polyacrylamide Criterion gels (Bio-Rad) and transferred onto nitrocellulose (0.1 μm pore size, BA79 Protran, Whatman, Maidstone, U.K.). Immunoblotting was performed as described (60) using mAbs to the cyt1 or cyt2 tails. CD46 fragments were detected using HRP-conjugated goat anti-mouse Abs and ECL and autoradiography. For experiments requiring increased sensitivity, a biotinylated secondary Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) and DyLight 800-conjugated streptavidin (Pierce, Rockford, IL) were monitored at 800 nm using the Odyssey Infrared Imaging System. Loading control input lysates were probed with anti–β-tubulin E7 mAb or anti-GAPDH rabbit mAb using secondary Abs conjugated to HRP or Infrared Dyes (680 or 800CW; Li-COR Biosciences, Lincoln, NE). Histogram stretches and contrast/brightness adjustments on scanned images were performed using Adobe Photoshop version 9.0, and the files transported to Adobe Illustrator for manuscript preparation. Data presented are representative of three or more individual independent experiments unless otherwise noted.

For quantifying CD46 fragments, End1 cells were infected as described previously with *N. gonorrhoeae* strain MS11 for 4 h at a multiplicity of infection (MOI) of 500. The levels of CD46 fragments in infected cells were determined by immunoprecipitation and immunoblotting as described previously. The levels of full length CD46 in mock-infected cells were determined similarly. CD46 fragment signals were expressed as a percentage of full length CD46 signals using Odyssey Infrared Imaging Systems software 2.1 (Li-COR Biosciences).

Results

CD46 cyt1 and cyt2 tail peptides are part of the normal cellular pool of CD46

CD46 is a type I transmembrane glycoprotein widely expressed on most nucleated human cells. The ectodomain of CD46 consists of four complement control protein repeats, an *O*-glycosylated serine-, threonine-, and proline (STP)-rich region (STP segments A, B, and C), and a 12-aa segment of unknown significance (Fig. 1*A*). CD46 terminates in one of two short cytoplasmic tails, cyt1 or cyt2, with unique sequences of 16 aa and 23 aa, respectively. Most cells express four abundant CD46 isoforms: C1, C2, BC1, and BC2, the products of alternative splicing of the STP-rich and cyt-coding regions (9).

We previously developed mAbs specific for unique epitopes in the cyt1 and cyt2 tails (29). We used these mAbs to immunoblot CHO cell lines stably expressing the CD46 C1, C2, BC1, and BC2 isoforms (61). The cyt1 mAb detected CD46 only in C1- and BC1-expressing cells, whereas the cyt2 mAb detected CD46 only in C2- and BC2-expressing cells (Fig. 1*B*, 1*C*). Both mAbs detected full-length CD46 (50-70 kDa) as well as a number of lower m.w. tail fragments. The cyt2 mAb also detected a band at ~43 kDa in all samples, however this band is not isolated by immunoprecipitation or affinity chromatography (Fig. 1*C*, data not shown). Immunoprecipitation facilitated the detection of low m.w. cyt2 peptides ~12 kDa

(Fig. 1*D*). GAPDH immunoblots demonstrated uniform sample loading (Fig. 1*E*) for panels B, C, and D. In summary, CD46-expressing CHO cells harbor full-length and low m.w. CD46 tail fragments. These fragments are not due to overexpression of CD46 in heterologous cells, as they are also detected in uninfected End1 human endocervical epithelial cells (Fig. 2) and by affinity chromatography (data not shown). These results suggest that CD46 low m.w. cyt1 and cyt2 fragments are part of the normal cellular pool of CD46 in human epithelial cells.

N. gonorrhoeae *induces the release of CD46 tail peptides*

N. gonorrhoeae recruits a number of host proteins, including CD46, to the cortex of epithelial cells beneath the site of attachment (55, 59, 62). These cortical plaques contain high concentrations of transmembrane, cytoskeleton, and adaptor proteins and are thought to act as signaling centers. More than 99.7% (SD ± 0.6) of cortical plaques stain with the CD46 ectodomain mAb, but only a fraction of them stain with the cyt1 (84.6%, SD ± 4.9) or cyt2 (3.4%, SD ± 1.3) tail-specific mAb (29). This suggests that some ectodomains are not connected to the tail. We therefore hypothesize that proteolytic cleavage of CD46 liberates the tail from the ectodomain.

As a first step toward testing this hypothesis, we determined whether low m.w. CD46 tail fragments accumulate in *Neisseria*-infected epithelial cells. End1 cells were infected with *N. gonorrhoeae* strain MS11 (P^+, Opa nonexpressing) for 4 h at various MOIs (Fig. 2). Cyt1 and cyt2 were immunoprecipitated from cell lysates and immunoblotted using the cognate mAbs. Infected cells had higher levels of a 9 kDa cyt1 peptide than mock-infected cells (Fig. 2*A*, *upper panel*) at all MOIs tested. In addition, cells infected at higher MOIs had higher levels of a 6 kDa cyt1 peptide than mock-infected cells or cells infected at lower MOIs. The 6 kDa cyt1 signal is more easily observed in the high contrast image of the same immunoblot (Fig. 2*A*, *middle panel*). Similar results were obtained for 12 kDa and 8 kDa cyt2 peptides (Fig. 2*B*). Immunoblots of input lysates for β-tubulin show that variation in tail peptide levels are not the result of variability in sample loading (Fig. 2*A*, 2*B*, *bottom panels*). These findings suggest that *Neisseria* stimulates CD46 processing, causing the accumulation of low m.w. cyt1 and cyt2 peptides in the host cell. As predicted for the CD46 tail isoforms, the cyt1 peptides are shorter than cyt2 fragments.

We determined the levels of CD46 fragments in *N. gonorrhoeae*-infected cells (see *Materials and Methods*). The cyt1 9 kDa fragment represents 3.6% (SD ± 1.8) of the full length CD46 signal in mock-infected cells. The cyt1 6 kDa fragment represents 0.11% (SD ± 0.02); the cyt2 12 kDa fragment represents 1.1% (SD ± 0.3); and the cyt2 8 kDa fragment represents 0.054% (SD ± 0.029).

Tfp and PilT are required for CD46 processing

N. gonorrhoeae Tfp are retractile fibers on the bacterial surface that promote adhesion and host cell signaling (18, 28, 53, 55, 63–66). Tfp retraction is mediated by PilT, the Tfp retraction motor (25). We determined whether Tfp and and *pilT* are required for CD46 processing. End1 cells were infected with wild type (wt) *N. gonorrhoeae* strain MS11, MS11-307 (a mutant that does not express Tfp and cannot adhere to cells), MS11*pilT* (a

pilT-null mutant), MS11*pilTi* (a mutant whose *pilT* can be induced by IPTG), and MS11*pilTi* and IPTG. Cell lysates were immunoprecipitated with the cyt1 or cyt2 mAb and immunoblotted using the same mAbs. The 6 kDa cyt1 and 8 kDa cyt2 peptides were detected in wt MS11-infected cells but not in cells infected with MS11-307, MS11*pilT* or MS11*pilTi* (Fig. 3). However, tail peptides were detected in cells infected with MS11*pilTi* in the presence of IPTG. This result suggests that Tfp and Tfp retraction are required for stimulating CD46 processing. Partial restoration of PilT activity by IPTG has been observed previously (64), and is likely due to IPTG not restoring PilT to wt levels in these cells (data not shown).

The matrix metalloproteinase inhibitor, GM6001, blocks release of CD46 cyt1 and cyt2 tail peptides

Several studies have demonstrated that CD46 can be cleaved by matrix metalloproteinase (MMP) (42–44). MMP cleavage of CD46 is believed to occur in the membrane proximal region of unknown function (43). We tested the hypothesis that *Neisseria* infection promotes MMP-dependent CD46 proteolysis. End1 epithelial cells were preincubated for 3 h in the presence of the broad spectrum MMP inhibitor GM6001 (20 μM), GM6001 nonfunctional analog (20 μM), or vehicle. Cells were then infected with *N. gonorrhoeae* strain MS11 for 4 h at an MOI of 500. Lysates were immunoprecipitated with the cyt1 or cyt2 mAb and immunoblotted using the same mAbs. Input lysates were blotted with a β-tubulin mAb to monitor loading.

Consistent with previous results, higher levels of the 6 kDa cyt1 and 8 kDa cyt2 tail peptides were detected in infected cells compared with uninfected cells (Fig. 4). Importantly, neither tail peptide was detected in cells infected in the presence of GM6001. The tail mAbs also precipitated 9 kDa cyt1 and 12 kDa cyt2 peptides from all conditions tested. GM6001 reduced these peptides to levels equivalent to, or lower than, that in vehicle-treated uninfected cells. The negative analog of GM6001 had no effect on *Neisseria*-induced accumulation of CD46 peptides. Consistent with this finding, flow cytometry experiments indicated that GM6001 partially inhibited infection-induced loss of CD46 surface staining (data not shown). In summary, these findings strongly suggest that MMPs play an important role in *Neisseria*-induced CD46 proteolysis.

PS/γ-S inhibitors block release of CD46 cyt1 and cyt2 tail peptides

PS/γS cleaves type I transmembrane proteins. Because CD46 is a type I transmembrane protein, we tested the hypothesis that it is processed by PS/γS. End1 cells and 16HBE14o- human bronchial epithelial cells were infected with *N. gonorrhoeae* strain MS11 for 4 h at an MOI of 500, in the presence of PS/γS inhibitors DAPT (10 μM), Compound E (200 nM), L-685–458 (1 μM), or vehicle. Lysates were immunoprecipitated with the cyt1 or cyt2 mAb and immunoblotted using the same mAbs. Input lysates were blotted with a β-tubulin mAb to monitor loading. Representative results from one of two experiments with 16HBE14o- cells are shown in Fig. 5*A*; the same results were obtained in End1 cells (data not shown). As expected, higher levels of the 6 kDa cyt1 and 8 kDa cyt2 tail peptides were detected in vehicle-treated infected cells compared with vehicle-treated uninfected cells. Importantly, neither tail peptide was detected in cells infected in the presence of PS/γS inhibitors.

The tail mAbs also precipitated a 9 kDa cyt1 and a 12 kDa cyt2 peptide from infected and uninfected cells. These larger tail peptides are likely to be the CD46C-terminal fragments, membrane-spanning segments containing a cytoplasmic tail but lacking an ectodomain. Higher amounts of C-terminal fragments were precipitated from inhibitor- than vehicle-treated cells (Fig. 5*A*), suggesting that blocking PS/γS activity led to their accumulation. Because PS/γS inhibition blocks release of the 6 kDa cyt1 and 8 kDa cyt2 peptides and causes the accumulation of the 9 kDa cyt1 and 12 kDa peptides, we propose that these larger tail fragments are precursors from which PS/γS liberates the smaller tail peptides. These results support a role for PS/γS in CD46 processing.

N. gonorrhoeae induced proteolytic processing of CD46-cyt1 and CD46-cyt2 in a PS/γS-dependent manner (Fig. 5*A*). We repeated the above experiment with *N. meningitidis*, a human pathogen closely related to *N. gonorrhoeae*. *N. meningitidis* strain 8013 induced the release of cyt1 and cyt2 tail peptides in 16HBE14o- cells and release was blocked by PS/γS inhibitors (Fig. 5*B*). Thus, two species of pathogenic *Neisseria* induce proteolytic processing of CD46-cyt1 and CD46-cyt2 in a PS/γS-dependent manner.

DN presenilin mutant cell lines do not process CD46

We also took a genetic approach to provide additional evidence for the involvement of PS/γS in processing CD46. Presenilin comprises the catalytic subunit of the PS/γS complex. The two major isoforms are termed PS1 and PS2. Cells expressing DN PS1 or PS2 mutant constructs cannot process APP or Notch (48, 67). We stably transfected 16HBE14o- cells with the PS1/PS2 DN mutant constructs or empty vector controls and obtained two presumptive positive PS1/PS2 DN mutant cell lines from two independent transfections. These cell lines were first tested for PS/γS activity using the Notch ΔE-6Myc reporter system (48). In an empty vector cell line, Notch ΔE-6Myc was cleaved into a 60 kDa Notch ICD, as expected. In contrast, very little Notch ICD was detected in the DN mutant cell lines (Fig. 6*A*). The DN mutant cell lines also failed to cleave a second reporter of PS/γS activity, the APP C99-6myc (data not shown). Thus, the 16HBE14o- PS1/PS2 DN cell lines have little detectable PS/γS activity.

Two 16HBE14o- PS1/PS2 DN cell lines were tested for their ability to process CD46. PS1/PS2 DN lines and empty vector control cells were mock-infected with medium or infected with *N. gonorrhoeae* MS11 or *N. meningitidis* 8013 at an MOI of 500 for 4 h. Lysates were immunoprecipitated with the cyt1 mAb and immunoblotted with the same mAb. A small amount of the 6 kDa cyt1 peptide was detected in mock-infected empty vector cells. As expected, the 6 kDa cyt1 peptide was present in higher amounts in *Neisseria*-infected empty vector cells. In constrast, the 6 kDa cyt1 peptide was barely detected in PS1/PS2 DN cells (Fig. 6*B*). Thus, cyt1 cleavage is blocked in PS1/PS2 DN cell lines.

Assays on the ability of PS1/PS2 DN cells to cleave CD46-cyt2 were inconclusive due to a high background level of the cyt2 peptide in mock-infected cells (data not shown). We cannot explain how the 8 kDa cyt2 peptide could be generated in PS1/PS2 DN cells. CD46-cyt2 cleavage may be caused by a distantly related presenilin homolog. There is evidence that presenilin exists in γ-secretase as a dimer (51). Incorporation of wt PS1 or PS2 into the γ-secretase complexes could explain the residual presenilin activity in these cells.

Nevertheless, our results, taken overall, demonstrate that the two CD46 isoforms are PS/γS substrates, and that two human pathogens, *N. gonorrhoeae* and *N. meningitidis*, stimulate PS/γS to cleave CD46.

Discussion

PS/γS regulates the function and signaling of many type I transmembrane proteins through proteolytic processing (30, 68). In this study, we have presented evidence that CD46-cyt1 and CD46-cyt2 isoforms are PS/γS substrates. Furthermore, PS/γS processing of CD46 is stimulated by *N. gonorrhoeae* and *N. meningitidis*. Based on these and other findings, we propose a model for CD46 processing by PS/γS (Fig. 7). *Neisseria* stimulates MMP(s) or other sheddases to cleave CD46-cyt1 and CD46-cyt2 at its juxtamembrane region, generating a soluble ectodomain and a membrane spanning C-terminal fragment. Subsequently, PS/γS cleaves the C-terminal fragment, releasing a 6 kDa cyt1 or 8 kDa cyt2 tail peptide into the cell. In accordance with established PS/γS nomenclature, we name these peptides ICD. We propose that these ICDs have biological functions (see below). Our model also predicts that PS/γS cleavage of the cyt1 and cyt2 C-terminal fragments will generate N-terminal stubs that remain in the extracellular space. We currently do not have the reagents to test this prediction. However, four substrates have been reported to yield extracellular peptides after PS/γS cleavage [reviewed in (31)]. Some of these fragments have important functions, most notably the amyloid β-peptide, a component of senile plaques in the brains of Alzheimer patients.

The site of PS/γS cleavage in the CD46 transmembrane domain is presently unknown. Many PS/γS-generated ICDs without N-terminal valines are difficult to detect without inhibition of the proteosome [reviewed in (69)]. Because we can detect CD46 ICDs without proteosome inhibition we speculate that the CD46 cyt1 and cyt2 ICDs have N-terminal valines. Two well-characterized PS/γS substrates, Notch and CD44, have cysteines in their transmembrane domains close to the cytoplasmic face near the established PS/γS cleavage sites. CD46 has a cysteine at position 313 near the cytoplasmic face of its transmembrane domain upstream of two valines. It is possible that PS/γS cleavage of CD46 occurs between Val314 and Val315.

N. gonorrhoeae-induced CD46 processing by PS/γS requires Tfp. Processing could be initiated by Tfp binding 1) directly to CD46, 2) to CD46 in conjunction with a host matrix protein, or 3) to another host component. There is precedence for the first two possibilities. Tfp has been shown to bind CD46 in vitro (26), and the group A streptococcal M protein binds CD46 in conjunction with $\alpha_v\beta_1$ integrin (70).

PilT, the Tfp retraction motor, is also required for CD46 processing, implying that mechanotransduction plays a role in this activity. The Tfp filament undergoes cycles of extension, substrate binding, and retraction. Tfp retraction exerts significant and biologically relevant forces on the substrate to which Tfp is attached (25, 71, 72). During retraction, Tfp tethered directly or indirectly to CD46 may induce conformational changes that expose previously buried protease cleavage sites. Several reports are interesting in this regard. On binding the adenovirus 11 knob protein, CD46 undergoes a conformational change that

uncovers previously hidden residues (73). Stretching of fibronectin also uncovers buried epitopes (74). Finally, indirect evidence suggests that mechanotransduction plays a role in PS/γS processing of Notch [reviewed in (75)].

N. gonorrhoeae infection downregulates CD46 in infected cells, and the cause of downregulation was hypothesized to be the release of $CD46^+$ vesicles (18, 28). When these studies were performed, cyt1 and cyt2 tail mAbs had not been constructed so shed CD46 was not characterized with respect to its cytoplasmic tail. It is possible that a portion of shed CD46 is the ectodomain released by MMP cleavage. The MMP and/or PS/γS cleavage may contribute to CD46 shedding and downregulation. Notably, *N. gonorrhoeae* infection induces expression of *Cyr61*, a mechanoresponsive extracellular matrix-associated protein that can induce expression of MMP1 and MMP3 (65, 76). We are currently working to identify specific MMPs that contribute to *Neisseria*-induced CD46 proteolysis.

Our model of PS/γS processing of CD46 could explain the very low level of CD46-cyt2 in the cortical plaque. PS/γS cleavage of CD46-cyt2 would free the tail from the ectodomain and membrane. Because cyt2 is present in cortical plaques much less frequently than cyt1, one might expect that cyt2 is cleaved more than cyt1. However, we detected robust PS/γS-dependent cleavage of both cyt1 and cyt2 isoforms. The frequent appearance of cyt1 in cortical plaques could mean that the PS/γS-liberated cyt1 peptide remains in the plaque through its association with cytoskeleton PDZ-containing adaptors via its FTSL sequence (77).

What are the functions of the cyt1 and cyt2 tails? The tails may have no function. Alternately, they may, like many PS/γS-generated ICDs, have a transcriptional role. The majority of PS/γS-generated ICDs traffic to the nucleus (30). The Notch and ErbB4 ICDs regulate transcription by binding to transcription factor complexes (40, 78, 79). Indeed, the Notch ICD functions at levels below the threshold of immunodetection (80). We are able to detect CD46 ICD and C-terminal fragment levels in infected cells and consistent with the Notch findings, they are present at low levels. These findings, and the presence of a consensus nuclear localization sequence in cyt1 and cyt2, lead us to propose that the cyt1 and/or cyt2 ICDs may influence transcription. In this context, *N. gonorrhoeae* affects the expression of >50 epithelial cell genes in a *pilT*-responsive manner (65). Cyt1 and/or cyt2 may play a role in regulating some of these genes.

The cyt1 and cyt2 tails may have signaling functions. Some PS/γS-liberated ICDs remain in the cytoplasm. Many functions have been described for cytoplasmic ICDs: cell adhesion, migration, and proliferation; golgi and mitochondrial trafficking; cell survival; cell junction remodeling; Rho activation; protein localization (β-catenin and tyrosinase); and src phosphorylation (30, 81). Some ICDs function in both the cytoplasm and nucleus. The ErbB4 ICD influences apoptosis signaling on the mitochondrial membrane as well as transcription (30). Both *N. gonorrhoeae* (27) and Ab ligation (7) stimulate cyt2 phosphorylation at tyrosine residue 354. The role of phosphorylation in CD46 function awaits clarification in our model.

It is important to consider the signaling potential of other CD46 cleavage products in addition to ICDs. Extracellular pools of shed CD46 may possess signaling capabilities. CD46 shed from cancer cells is known to retain complement regulatory activity (43). Shed CD46 may coat adherent *N. gonorrhoeae* and confer resistance to complement killing. The CD46 C-terminal fragment generated by MMP cleavage may also play a role in propagating signals. This function has been demonstrated for the C-terminal fragment of DCC (deleted in colorectal carcinoma), another PS/γS substrate. The DCC C-terminal fragment retains activity in neurite outgrowth assays, whereas the cytosolic ICD fragment does not. In this case, PS/γS cleavage of DCC attenuates a signal being sent by the C-terminal fragment (82).

In summary, CD46 is a multifunctional immunoregulatory protein that is a target of many pathogens. Little is known about the mechanisms by which the CD46 cyt1 and cyt2 tails propagate signals. The identification of CD46 as a PS/γS substrate, and the findings that pathogenic *Neisseria* stimulate its processing by PS/γS, provide a starting point for dissecting CD46 signaling pathways, a critical step toward understanding the full range of functions of CD46.

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Abbreviations used in this paper

APP	amyloid precursor protein
CHO	Chinese hamster ovary
CTF	C-terminal fragment
cyt	cytoplasmic tail
DCC	deleted in colorectal carcinoma
DN	dominant negative
End1	a human endocervical cell line
IB	immunoblotting
ICD	intracellular domain
IP	immunoprecipitation
IPTG	isopropyl-β-D-thiogalactopyranoside
MMP	matrix metalloproteinase
MOI	multiplicity of infection

N	N-linked oligosaccharides
O	*O*-linked oligosaccharides
PS/γS	presenilin γ-secretase
PS1	presenilin 1 isoform
PS2	presenilin 2 isoform
STP	serine, threonine, and proline
Tfp	type IV pili
Tr1	T regulatory type 1
U	unknown function
V	vector
wt	wild type

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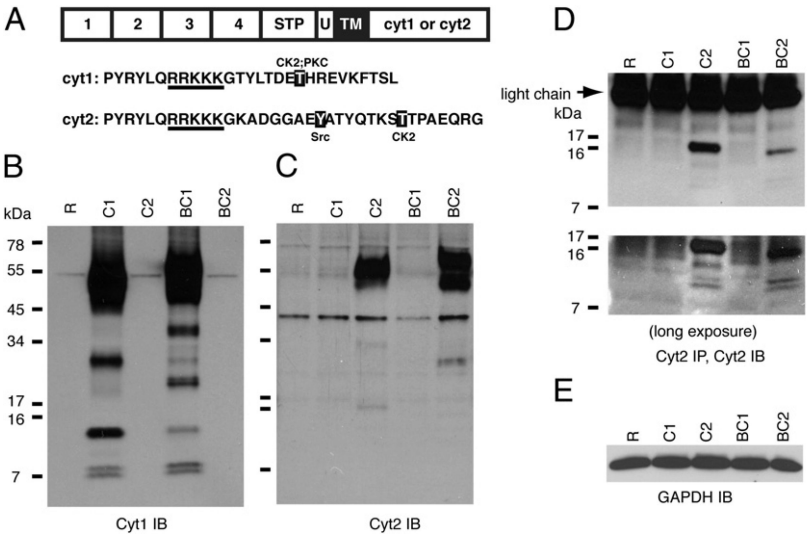

FIGURE 1.

Low m.w. CD46 tail fragments accumulate in CHO cells stably expressing CD46 isoforms. *A*, Illustration of CD46 structure: the ectodomain consists of four complement control protein repeats (1–4) an alternatively spliced STP-rich region, and a small segment of unknown function (U). The transmembrane segment (TM) is followed by one of two cytoplasmic tails (cyt1 or cyt2). Known and predicted tail phosphorylation sites are in black boxes; putative nuclear localization sequences are underlined. *B* and *C*, CHO cell lysates were resolved by SDS-PAGE and immunoblotted with a mAb specific to the cyt1 or cyt2 tail (29). *D*, immunoprecipitation (IP) of CHO cell lysates using the cyt2-specific mAb, followed by immunoblotting of the precipitates using the cyt2 mAb. *E*, As a loading control, GAPDH levels in lysates used for immunoblotting (IB) (*B* and *C*) and IP and IB (*D*) were determined with anti-GAPDH mAb. R: Parent CHO cell line; C1, C2, BC1, and BC2: CHO lines expressing the different CD46 isoforms. IB, immunoblotting; IP, immunoprecipitation; STP, serine, threonine, and proline; TM, transmembrane; U, unknown function.

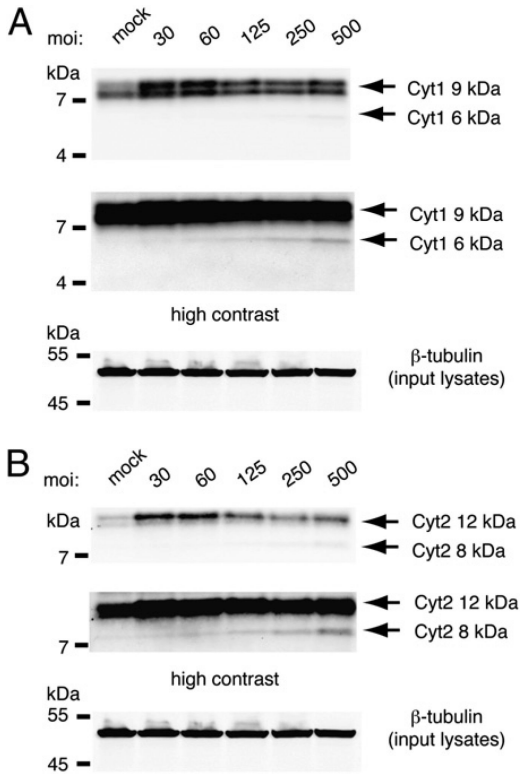

FIGURE 2.

N. gonorrhoeae infection of endocervical cells induces cleavage of CD46-cyt1 and CD46-cyt2 isoforms. End1 human endocervical epithelial cells were infected with strain MS11 at various MOI for 4 h, or mock infected with medium, and cyt1 or cyt2 tail peptides were immunoprecipitated with the cyt1 (*A*) or cyt2 (*B*) mAb and immunoblotted using the same mAb. *Middle panels*: High contrast image of the same immunoblot to illustrate the lower M_r bands. *Bottom panels*: Loading controls; input lysates were immunoblotted with anti–β-tubulin mAb.

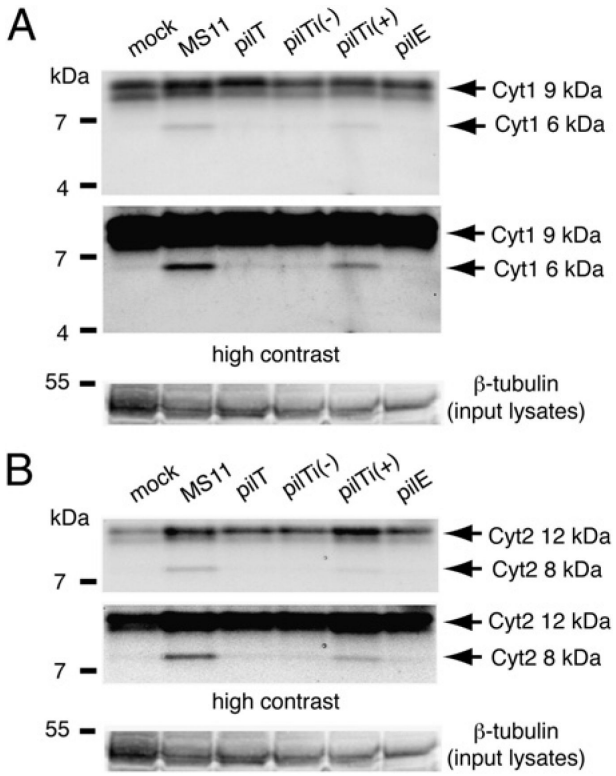

FIGURE 3.

N. gonorrhoeae stimulates CD46 processing in a Tfp- and *pilT*-dependent manner. End1 cells were mock infected with medium or infected with wt MS11, MS11*pilT*, MS11*pilTi*, MS11*pilTi* + IPTG (4 mM), or MS11*pilE* for 4 h at an MOI of 500. Cell lysates were immunoprecipitated with the cyt1 (*A*) or cyt2 (*B*) mAb and immunoblotted using the same mAb. High and low contrast images of each immunoblot are presented. *Bottom panels*: Loading controls; input lysates were immunoblotted with anti–β-tubulin mAb.

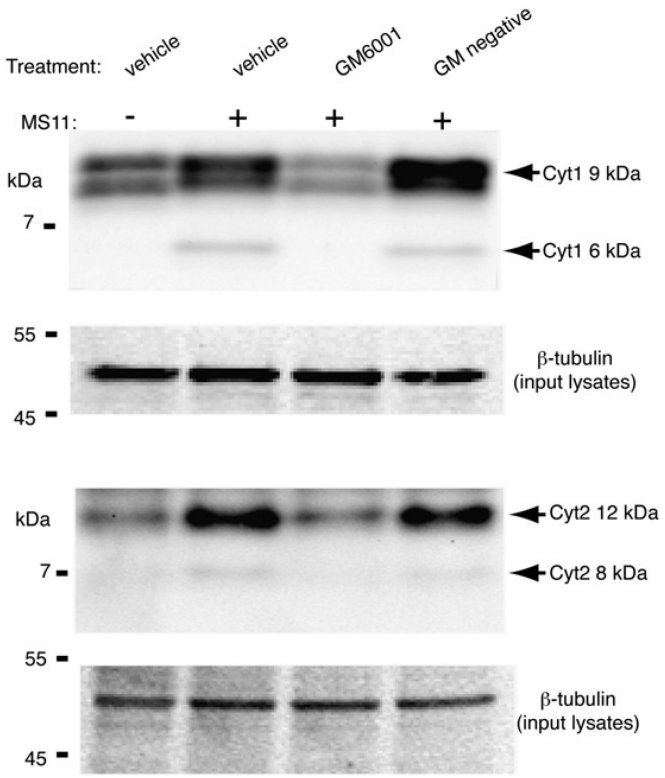

FIGURE 4.

MMP inhibitor GM6001 prevents release and accumulation of CD46 tail peptides in *Neisseria*-infected cells. End1 cells were infected with *N. gonorrhoeae* strain MS11 in the presence or absence of MMP inhibitor GM6001 (20 μM), GM6001 negative analog (GM negative, 20 μM), or vehicle. Cyt1 and cyt2 tail peptides were immunoprecipitated from the lysates then immunoblotted with the appropriate mAb. *Upper panels*: cyt1 immunoblots and loading control. *Bottom panels*: cyt2 immunoblots and loading control. Loading controls: input lysates immunoblotted with the anti–β-tubulin mAb.

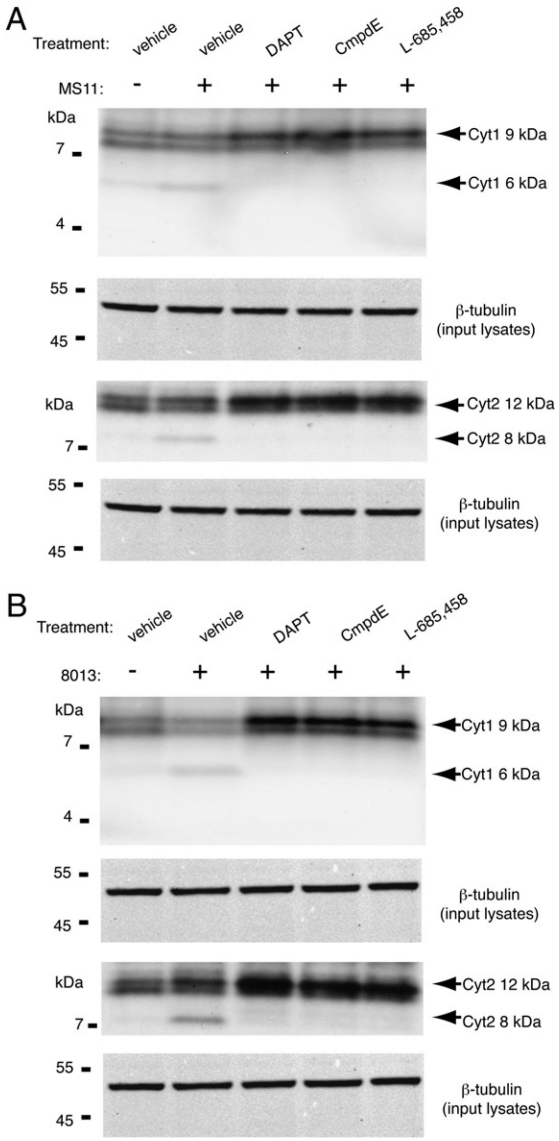

FIGURE 5.

PS/γS inhibitors prevent release and accumulation of CD46 tail peptides in *Neisseria*-infected cells. 16HBE14o- cells were infected with *N. gonorrhoeae* strain MS11 (*A*) or *N. meningitidis* strain 8013 (*B*) (MOI of 500, 4 h) in the presence or absence of PS/γS inhibitor DAPT (10 μM), Compound E (200 nM), or L-685-458 (1 μM). Cyt1 and cyt2 tail peptides were immunoprecipitated from the lysates then immunoblotted with the appropriate mAb. *Upper panels*: cyt1 immunoblots and loading control. *Bottom panels*: cyt2 immunoblots and loading control. Loading controls: input lysates immunoblotted with the anti–β-tubulin mAb.

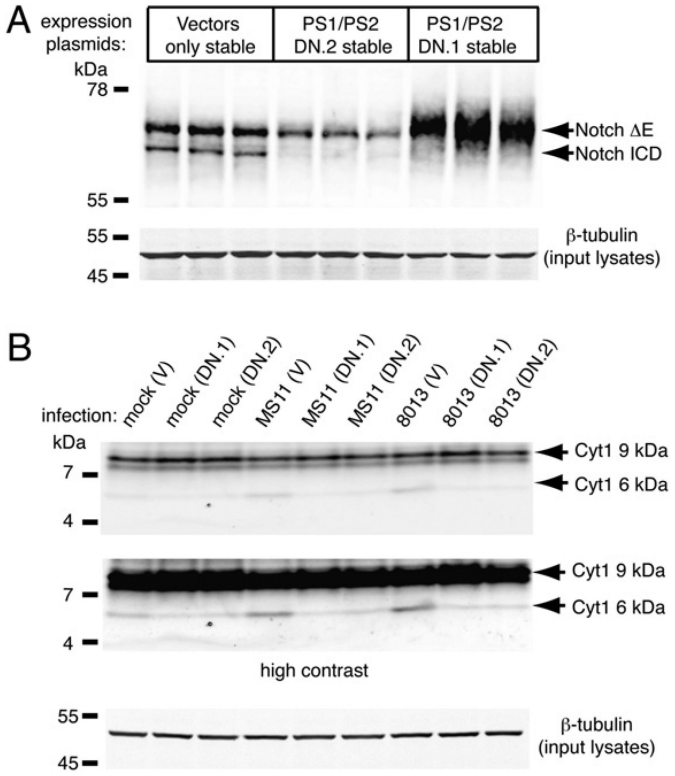

FIGURE 6.

DN presenilin mutations block release and accumulation of CD46 tail peptides. *A*, A 16HBE14o- cell line stably expressing empty vector controls and two 16HBE14o- DN PS1/PS2 cell lines (DN.1 and DN.2) were transiently transfected with the Notch ΔE-6MT (Notch ΔE) PS/γS activity reporter. Notch ΔE and Notch ICD were detected by immunoblotting total cell lysates with an Ab to the myc tag at the C-terminal end of the fusion protein. Notch reporter transfections were performed in triplicate for each cell line. *B*, 16HBE14o- DN PS1/PS2 cell lines(DN.1 and DN.2) and control cells stably expressing empty vectors (V) were mock infected with medium or infected with *N. gonorrhoeae* MS11 or *N. meningitidis* 8013 (MOI of 500, 4 h). Cyt1 tail peptide was immunoprecipitated from cell lysates then immunoblotted using the cyt1 mAb. *Bottom panels*: Loading control; input lysates immunoblotted with the anti–β-tubulin mAb.

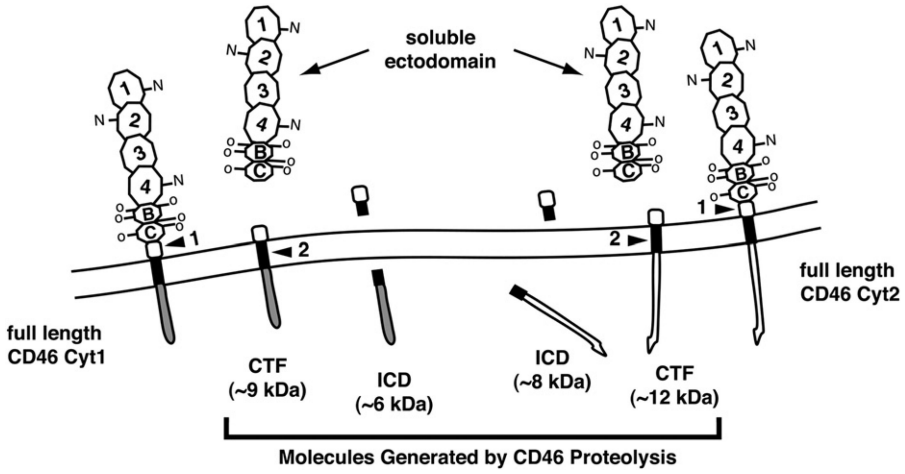

FIGURE 7.

Model for PS/γ-S processing of CD46-cyt1 and CD46-cyt2. 1: MMPs cleave the cyt1 (left cartoon, gray tail) and cyt2 (right cartoon, white tail) juxtamembrane regions of CD46, generating soluble ectodomains and a ~9 kDa membrane-spanning cyt1 C-terminal fragment (CTF) and a ~12 kDa cyt2 CTF. 2: PS/γS cleaves the CTF TM segments, releasing a ~6 kDa cyt1 ICD and a ~8 kDa cyt2 ICD from the cell membrane. Based on what is known about other PS/γS substrates, we predict that the small peptides generated by PS/γS cleavage of the CTFs remain in the extracellular milieu. Numbered octagons: Complement control protein repeats. Octagons B and C: STP-rich segments. White rectangle: juxtamembrane 12 aa segment. Black rectangle: transmembrane segment. Figures are not drawn to scale. N, N-linked oligosaccharides. O, *O*-linked oligosaccharides.