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Monoclonal Antibody Detection of CD46 Clustering beneath *Neisseria gonorrhoeae* Microcolonies

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CD46 (membrane cofactor protein), a complement-regulatory protein that participates in innate and acquired immunity, also serves as a receptor for viral and bacterial pathogens. CD46 isoforms terminate in one of two cytoplasmic tails, Cyt1 or Cyt2, which differ in signaling and trafficking properties. Dissecting the functions of the two cytoplasmic tails in these cellular processes has been hampered by the absence of specific reagents. Here we report the construction of Cyt1- and Cyt2-specific monoclonal antibodies (MAbs). These MAbs recognize unique epitopes within the tails and can be used for immunofluorescence microscopy, immunoblotting, and immunoprecipitation. Studies of *Neisseria gonorrhoeae*-infected cells with the CD46 tail MAbs demonstrate the differential recruitment of Cyt1 and Cyt2 to the cortical plaque.

CD46 has an established role in innate immunity as a complement-regulatory protein. It prevents the inappropriate deposition of complement on autologous cells by acting as a cofactor for factor I-mediated cleavage of C3b and C4b (reviewed in reference 24). Cellular immunity is also influenced by the ability of CD46 to promote T-cell costimulation and proliferation (38), enhance antigen presentation (37), regulate the contact-type hypersensitivity response (30), and promote the development of Tr1 cells (18). CD46 plays a role in reproduction, transplant rejection, and cancer biology (5, 36, 39). The following human pathogens can utilize CD46 as a receptor: measles virus (3, 33), human herpesvirus 6 (40), adenovirus subgroups B and D (6, 41, 47), and *Streptococcus pyogenes* (34). Type IV pili from pathogenic neisseriae can associate with CD46 and it has been proposed that CD46 serves as a receptor for *Neisseria gonorrhoeae* and *Neisseria meningitidis* (17).

CD46 is a type I membrane glycoprotein. The ectodomain of CD46 contains four tandem complement control protein modules, an O-glycosylated serine-, threonine-, and proline-rich region (STP segments A, B, and C), and a 12-amino-acid segment of unknown significance. Alternative splicing of the STP- and the cytoplasmic tail-coding regions of the mRNA generates four major isoforms, C1, BC1, C2, and BC2; all four forms are found in most cells (43). The two cytoplasmic tails share a common membrane-proximal sequence and unique sequences of 16 and 23 amino acids for Cyt1 and Cyt2, respectively (Fig. 1A).

Both tails negatively affect replication of measles virus (Edmonston strain) in CD46-transfected murine macrophages, whereas tailless CD46 constructs cause an increase in replication (13). Cyt1 and Cyt2 isoforms expressed in CHO cells can support adhesion of pathogenic neisseriae (17) but Cyt1 tails with deletion mutations do not (16). Both tails have the ability to associate with macrophage tyrosine kinases and be tyrosine phosphorylated by macrophage lysates (46). Cyt2 tyrosine phosphorylation has been linked to the src kinases Lck and c-Yes in response to antibody cross-linking of Jurkat T cells (45) and neisserial infection of epithelial cells, respectively (22).

Much of our knowledge of Cyt1 and Cyt2 trafficking and signaling is derived from studies of CD46 expression in nonhuman cell lines (12, 26, 28, 29) or CD46 transgenic mouse cells (30). Ectodomain antibodies cannot distinguish Cyt1 and Cyt2 isoforms since their migration patterns overlap on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gels. Assigning functions to Cyt1 and Cyt2 isoforms has been hampered by the lack of tail-specific monoclonal antibodies (MAbs). We report the development of MAbs that bind specifically to the Cyt1 and Cyt2 cytoplasmic tails of CD46.

Synthetic peptides (Global Peptide Services) (Fig. 1A) conjugated via a Cys-Gly linker to keyhole limpet hemocyanin were used to make MAbs for the Cyt1 and Cyt2 cytoplasmic tails of CD46 according to standard procedures (11). Antibodies were isotyped using an IsoStrip kit (Roche Applied Science) as directed by the manufacturer. Both clones are immunoglobulin G1 (IgG1) and have kappa light chains. To demonstrate the specificity of each MAb for its cognate CD46 tail peptide, enzyme-linked immunosorbent assay (ELISA) was performed using protein A-agarose-purified antibodies (Fig. 1) (1). Both MAbs 2F1 (Fig. 1B, anti-Cyt1) and 13G10 (Fig. 1C, anti-Cyt2) reacted specifically with their cognate peptides but not the control peptide RhUS2, a cytomegalovirus sequence. FN18, an isotype-matched MAb specific for rhesus CD3 antigen, did not react with either of the CD46 tail peptides or a control rhesus CMV US2 peptide (Fig. 1D). At high concentrations, MAb 2F1 reacted slightly with both noncognate peptides tested and blank wells (Fig. 1B and data not shown). This background was significantly reduced using alternative means of purifying the 2F1 antibody that avoided low-pH exposure, suggesting that denatured or aggregated antibody might be the cause (data not shown).

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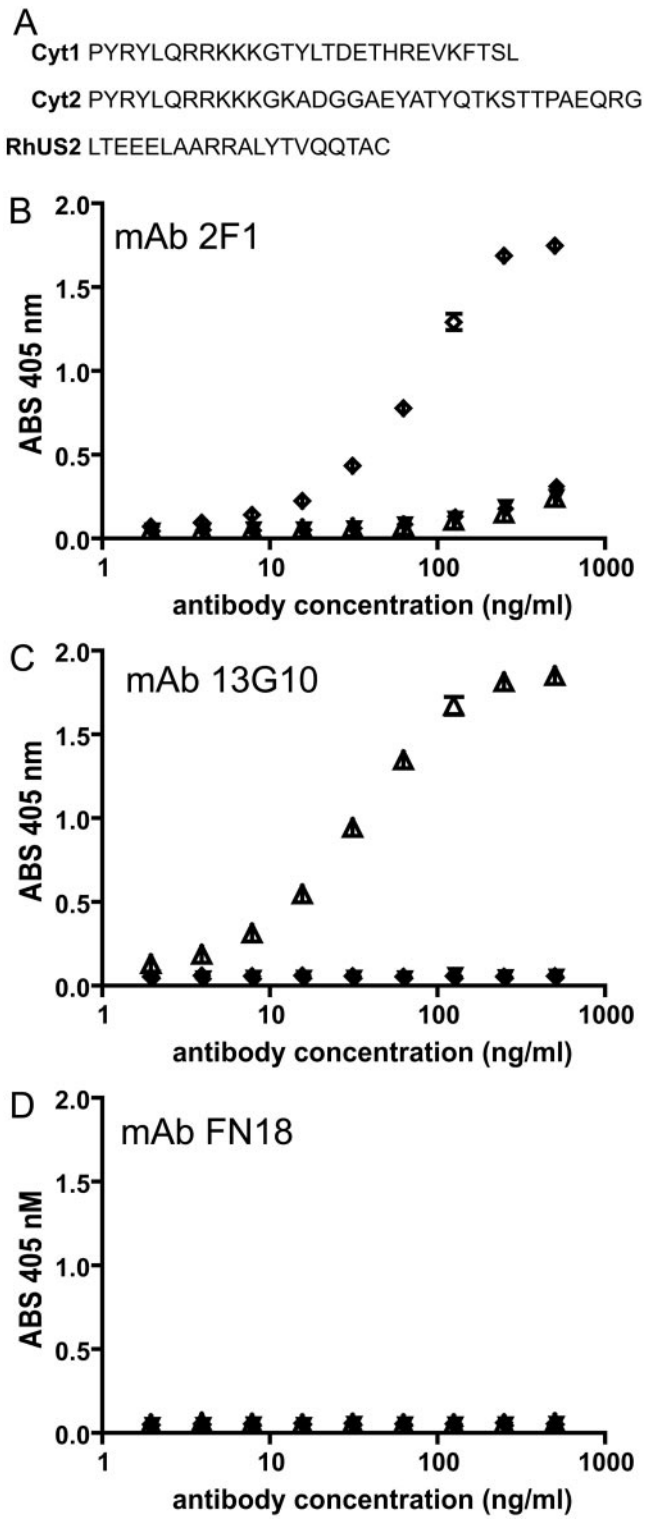

FIG. 1. ELISA characterization of CD46 tail-specific monoclonal antibody binding. A fixed concentration of each of three peptides (A) was immobilized in microtiter wells, and the binding to these peptides by Cyt1 MAb 2F1 (B) and Cyt2 MAb 13G10 (C) was determined. An isotype-matched MAb, FN18, which recognizes rhesus CD3 antigen, served as the negative control (D). Peptide symbols: Cyt1, open diamonds; Cyt2, open triangles; RhUS2, solid inverted triangles. Each antibody concentration tested was plotted as the mean ± the standard deviation from one representative experiment.

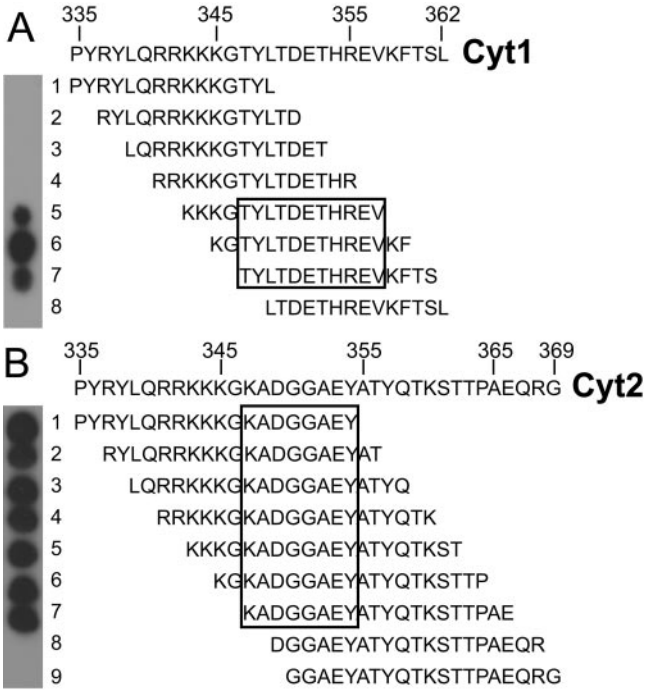

FIG. 2. Mapping of the epitopes recognized by Cyt1 MAb 2F1 and Cyt2 MAb 13G10. Overlapping peptides containing Cyt1 (A) or Cyt2 (B) tail sequences were probed with their cognate MAb (leftmost panels). The core epitope sequence recognized by each MAb is boxed. The numbering of the peptide residues is based on the predicted amino acid sequences of the CD46 C1 and C2 isoform cDNA clones H2-14 and H2-15 (35).

Because of the short length of the CD46 cytoplasmic tails, we reasoned that the tail-specific MAbs might recognize linear epitopes. Oligopeptides synthesized on activated cellulose membranes (kindly provided by Donelson Smith or purchased from Sigma Genosys) were used to map the core epitope regions of each CD46 tail MAb. Interacting peptides were identified by immunodetection (Fig. 2) according to an established protocol (20). Each antibody recognized a unique portion of its cognate peptide, demonstrating the specificity of MAb 2F1 for Cyt1 and MAb 13G10 for Cyt2. These experiments were performed twice with identical results. Secondary antibody-only controls did not react with any of the peptides (data not shown).

CD46 is expressed on all nucleated human cells but not red blood cells (42). To further characterize the specificity of the Cyt1 and Cyt2 tail MAbs, we tested their performance as reagents for immunoblotting. Cells were lysed in 50 mM Tris-Cl (pH 7.2), 0.15 M NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 5 mM EDTA containing 264 μM sodium orthovanadate, 50 mM NaF and a protease inhibitor cocktail (Roche). Lysates were separated by 10% SDS-PAGE under reducing conditions and transferred to nitrocellulose. Antibodies specific for the CD46 ectodomain (Santa Cruz Biotechnology) and cytoplasmic tails were used to probe lysates (20 μg/lane) of eight human cell lines, A431 (ATCC CRL-1555), Chang (ATCC CCL-20.2), End1 (4), 16HBE14o$^-$ (2), HCjE (9), Hec1B (ATCC HTB-113), ME180 (ATCC HTB-33), and T84 (ATCC CCL-248), and two nonhuman cell lines, LLC-PK1 (ATCC CL-101, porcine kidney) and MDCK (ATCC CCL-34, canine kidney) (Fig. 3). CD46 was detected only in the

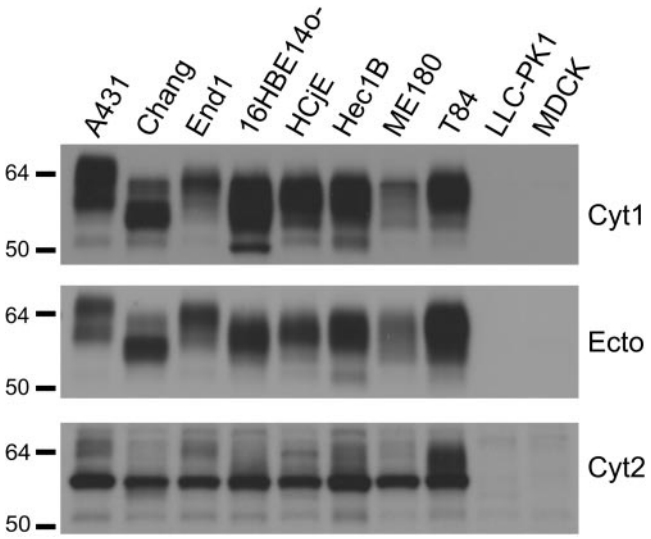

FIG. 3. Immunoblot detection of CD46 in epithelial cell lines using Cyt1 MAb 2F1 and Cyt2 MAb 13G10. Total cell lysates (20 μg/lane) from ten epithelial cell lines were separated by SDS-PAGE and transferred to nitrocellulose. The blot was successively probed with Cyt1 MAb 2F1 (top panel), ectodomain polyclonal antibody H-294 (middle panel), and Cyt2 MAb 13G10 (bottom panel). The first eight lanes contain lysates from human cell lines; the last two lanes are derived from porcine (LLC-PK1) and canine (MDCK) cells. Sizes are shown on the left in kilodaltons.

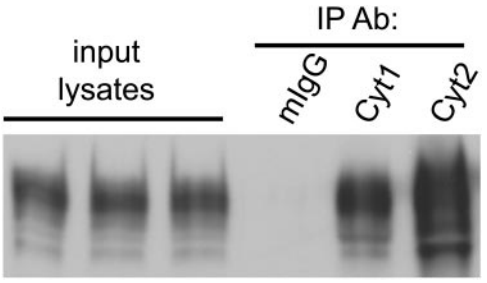

FIG. 4. Immunoprecipitation (IP) of CD46 by Cyt1 MAb 2F1 and Cyt2 MAb 13G10 from T84 cell lysates. A normal mouse IgG antibody (mIgG; Santa Cruz Biotechnology) served as the negative control. Input lysates used for the immunoprecipitations were also probed with the ectodomain polyclonal antibody (Ab) H-294 (leftmost three lanes) to demonstrate equal levels of CD46 in all samples.

human cell lines with antibodies to the ectodomain and both cytoplasmic tails (Fig. 3). Cyt1 and Cyt2 peptides (Fig. 1) abolished the immunoreactivity of its cognate MAb and had no effect on the heterologous MAb (data not shown). CD46 was not detected in human red blood cells by either ectodomain or tail MAbs (data not shown).

ME180 cells had the lowest amounts of Cyt1 of the cell lines tested. This correlates with the CD46 mRNA expression pattern in ME180 cells, in which the Cyt2 isoforms BC2 and C2 were detected at much higher levels than the Cyt1 isoforms BC1 and C1 (44, 45). CD46 typically migrates as a broad doublet ranging from 51 to 68 kDa under reducing conditions on SDS-PAGE (25, 35). Doublets were detected by the Cyt1-, Cyt2-, and ectodomain-specific antibodies in eight human cell lines tested by varying the exposures times (data not shown). Although the Cyt2 MAb 13G10 detected doublets in the expected size range, it primarily detected a lower-molecular-mass band of 58 to 59 kDa (Fig. 3). The basis for this reactivity is unclear.

MAb 13G10 may have a higher affinity for a lower-molecular-mass CD46 isoform (which would likely be C2). Alternatively, this result may reflect posttranscriptional regulation of the CD46 isoforms. In ME180 cells, BC2 mRNA levels are higher than C2 levels (44, 45). Finally, MAb 13G10 might recognize a low-molecular-mass pool of Cyt2 isoforms that have not been fully glycosylated. In addition to the typical CD46 broad doublet, an immunoreactive band near 50 kDa was detected by both CD46 tail MAbs in some cell lines (Fig. 3). The identity of this band is unknown, but the band has been observed in other studies with CD46 ectodomain antibodies (45).

Both 2F1 (anti-Cyt1) and 13G10 (anti-Cyt2) successfully immunoprecipitated CD46 from T84 cell lysates, whereas a control mouse antibody did not (Fig. 4). Input lysates blotted with a polyclonal antibody to the CD46 ectodomain demonstrated that equal levels of CD46 were present in each lysate. Polyclonal antibodies to the CD46 ectodomain immunoprecipitated both Cyt1 and Cyt2 isoforms whereas control rabbit and goat antibodies did not (data not shown).

CD46 localization in polarized cells. CD46 is found primarily on the basolateral membranes of polarized epithelial cells (28). We determined the location of the CD46 ectodomain and Cyt1 and Cyt2 tails in polarized T84 human colonic carcinoma cells using confocal microscopy (15). The acquisition system (Laser Sharp) used a krypton/argon laser with excitation lines at 488, 568, and 647 nm. Sequential detection was used with three eight-bit channels (photomultiplier tube detectors) to acquire stacks (0.5-μm Z steps) across a folded transmembrane filter to achieve improved *X-Z* resolution using a Nikon Plan Apo 60X (NA 1.40) oil immersion objective. The images were captured on a Bio-Rad 1024 ES laser scanning confocal imaging system attached to an inverted Nikon Eclipse TE 300 microscope. Indirect immunofluorescence was used to visualize CD46 (ectodomain, Cyt1, or Cyt2) and a tight junction marker, ZO-1 (Zymed Laboratories), was used to locate the boundary between the apical and basolateral membranes. The DNA dye Draq5 (Alexis Biochemicals) was used to visualize nuclei. CD46 ectodomain MAb J4.48 (Beckman Coulter) predominantly stained the basolateral membranes of polarized T84 cells (Fig. 5).

Cyt1 MAb 2F1 primarily stained basolateral membranes and the cytosol (Fig. 5). Cyt2 MAb 13G10 stained both apical and basolateral membranes in addition to the cytosol (Fig. 5). Secondary-antibody-only controls did not react with the cells (data not shown).

CD46 clusters beneath adherent neisseriae. Upon infection with *N. gonorrhoeae*, a number of epithelial cell proteins are recruited to the site of bacterial attachment (14, 22, 23, 31, 32), forming structures termed cortical plaques. CD46 can bind type IV pili of pathogenic neisseriae (16, 17). Using CD46-green fluorescent protein (GFP) fusions, Gill et al. recently demonstrated the clustering of CD46 isoforms beneath adherent neisseriae (8). Deconvolution immunofluorescence micros-

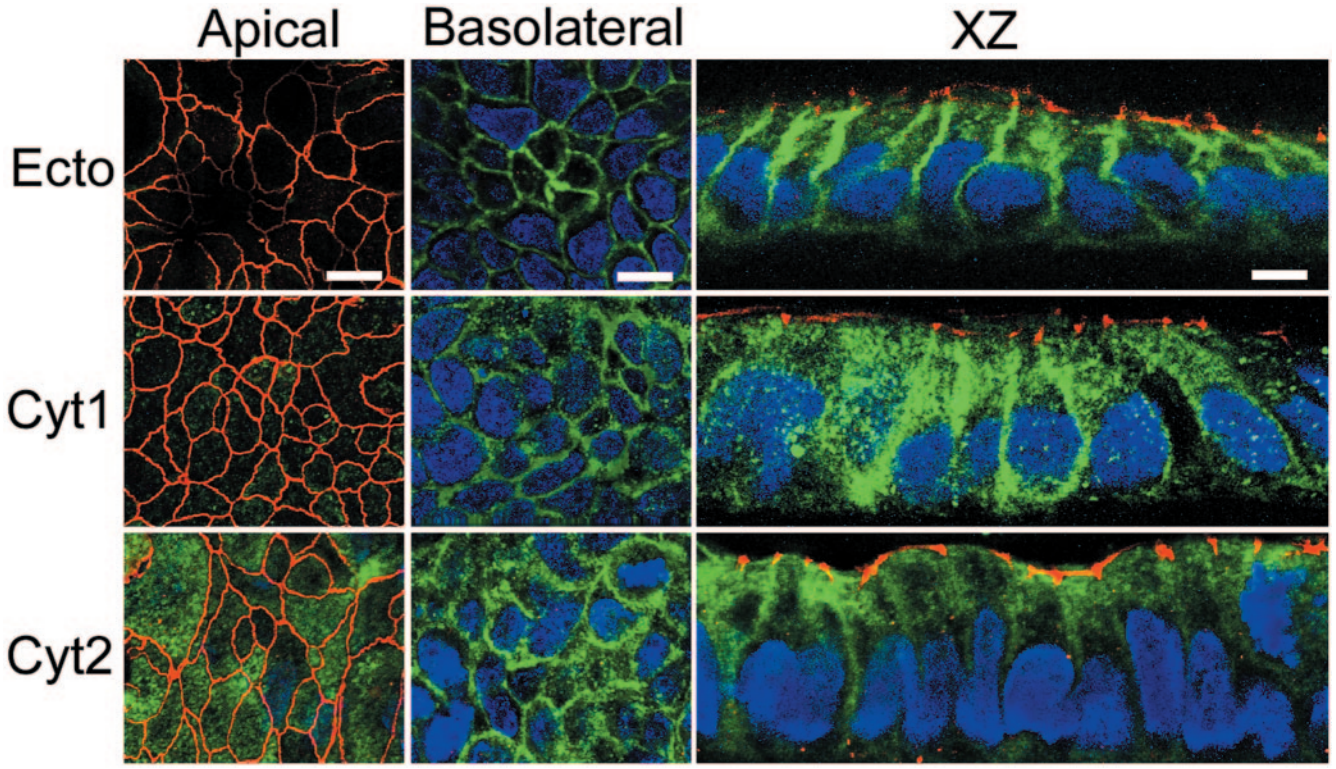

FIG. 5. Immunofluorescence microscopy of CD46 using Cyt1 MAb 2F1 and Cyt2 MAb 13G10. Polarized, uninfected T84 human epithelial cells were fixed and stained with Draq5 to visualize nuclei (blue signal), anti-ZO1 polyclonal antibodies to visualize tight junctions (red signal), and an MAb to CD46 ectodomain, Cyt1, or Cyt2 (green signal). The leftmost panels show *X-Y* sections from the apical regions of the monolayers in the proximity of the tight junction. The middle panels show *X-Y* sections from the basal region of the monolayers. The rightmost panels show *X-Z* (transverse) sections of the monolayers. Images were acquired using the Restoration microscopy system (API, Issaquah, WA) fitted with Deltavision Softworx software and imported to Adobe Photoshop v. 7.0.1 for manuscript preparation. Scale bars, 10 μm.

opy was performed as described previously (23) to determine the location of CD46 in *N. gonorrhoeae*-infected nonpolarized epithelial cells. End1 (Fig. 6) and Hec1B human epithelial cells were infected for 3 hours with strain MS11 using a multiplicity of infection of 50. The clustering of phosphorylated ezrin/radixin/moesin (pERM), a known cortical plaque component (23, 31), beneath microcolonies was first confirmed. As shown in Table 1, pERM clustered beneath nearly all adherent microcolonies.

Next, pERM-positive plaques were scored for the clustering of the CD46 ectodomain, Cyt1, and Cyt2. Nearly 100% of the pERM-positive clusters also contained high concentrations of CD46 ectodomain, as judged by staining with MAb J4.48 (Fig. 6A and Table 1). Similar observations were obtained with two other CD46 ectodomain MAbs, MEM-258 and Huly-m5 (data not shown). Cyt1 clustering was detected in over 80% of pERM-positive plaques in End1 and Hec1B cells (Fig. 6B and Table 1).

In contrast to the CD46 ectodomain and Cyt1, the Cyt2 signal was observed rarely in association with pERM clusters in End1 cells, and not at all in Hec1B cells (Table 1). The Cyt2 signal was less intense at the site of the cortical plaques than in other regions of the infected cell (Fig. 6C). However, Cyt2 did aggregate at the periphery of the microcolonies. In summary, the CD46 ectodomain and Cyt1 tail clustered at high levels beneath *N. gonorrhoeae* microcolonies, while the Cyt2 tail did not. These phenotypes were also observed in two additional human epithelial cell lines, T84 and HCjE (data not shown).

In this paper, we report the development of monoclonal antibodies to the CD46 cytoplasmic tails Cyt1 and Cyt2. The MAbs were found to be specific for their cognate tails by ELISA (Fig. 1), immunoblotting (Fig. 3), and immunoprecipitation (Fig. 4). The MAbs 2F1 (anti-Cyt1) and 13G10 (anti-Cyt2) recognize epitopes unique to each tail sequence (Fig. 2).

The CD46 ectodomain and Cyt1 tail were clustered in neisseria-induced cortical plaques. In contrast, Cyt2 was found in very few pERM-positive plaques, and only in one of two cell lines tested (Table 1). Cyt2 appears, however, to be recruited to the edges of adhered microcolonies but absent from the pERM-positive portion of the plaque (Fig. 6C). We are currently addressing the lack of Cyt2 immunostaining in cortical plaques.

A number of explanations could account for the absence of the Cyt2 tail in neisseria-induced cortical plaques. First, CD46 Cyt2 isoforms may be exclusively recruited to the periphery of adhered microcolonies. Second, Cyt2 isoforms may be selectively shed from the plaques. Gill et al. recently demonstrated that neisseriae induce infected cells to secrete CD46 (7). The study did not establish which CD46 isoforms are secreted, although it showed that over the course of infection surface and intracellular CD46 levels dropped while mRNA levels did not. Third, Cyt2 isoforms

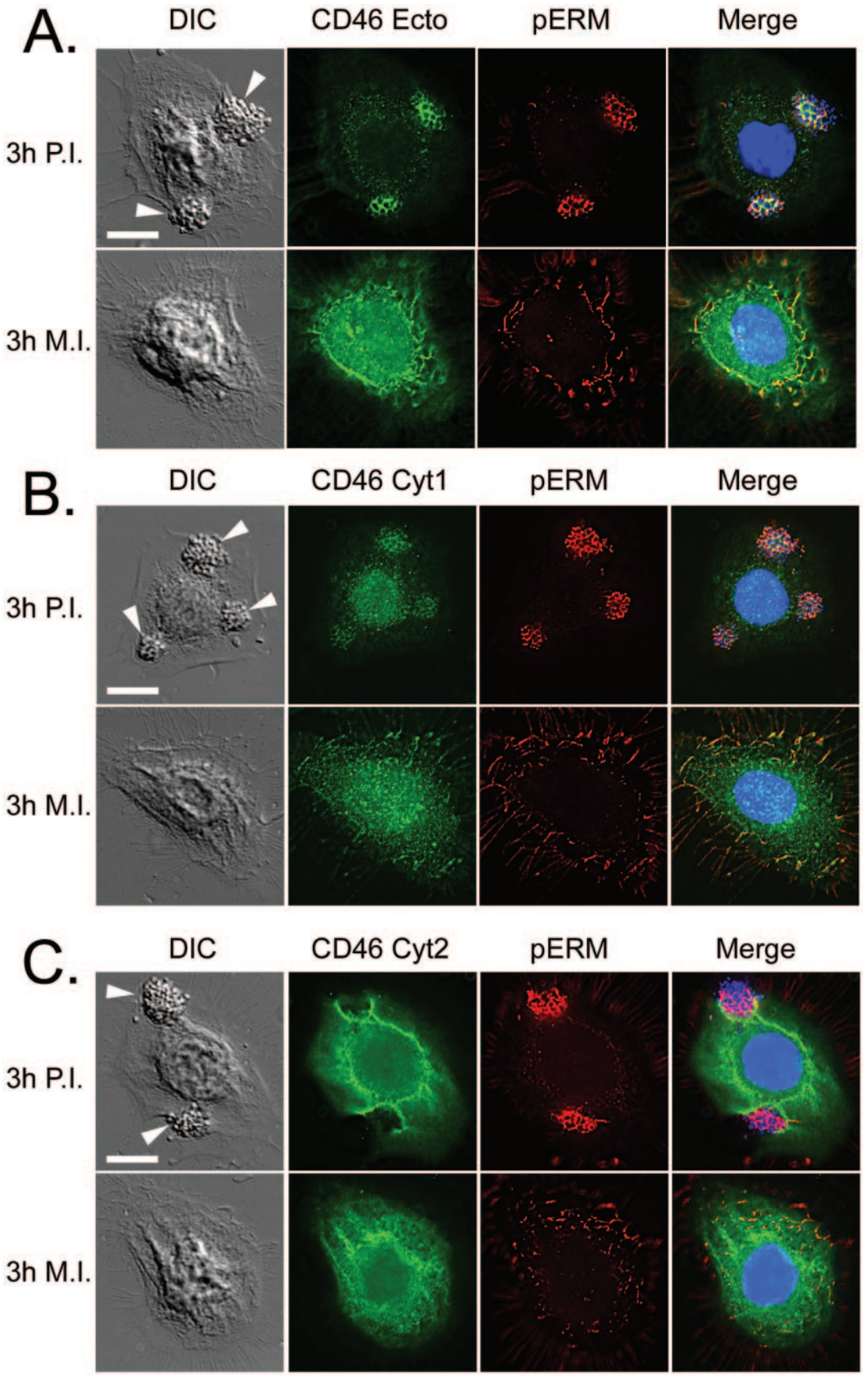
A.
DIC
CD46 Ecto
pERM
Merge
3h P.I.
3h M.I.
B.
DIC
CD46 Cyt1
pERM
Merge
3h P.I.
3h M.I.
C.
DIC
CD46 Cyt2
pERM
Merge
3h P.I.
3h M.I.

TABLE 1. Quantitation of colocalization of CD46 ectodomain, Cyt1, and Cyt2 with pERM in cortical plaques[a]

Cells	Mean % of colonies ± SD			
	pERM/DAPI[b] (*n*)	Ecto/pERM[c] (*n*)	Cyt1/pERM[c] (*n*)	Cyt2/pERM[c] (*n*)
End1	99.3 ± 1.2 (318)	99.7 ± 0.6 (328)	84.6 ± 4.9 (284)	3.4 ± 1.3 (323)
Hec1B	98.8 ± 1.1 (329)	96.0 ± 3.6 (324)	81.6 ± 16 (316)	0 ± 0 (326)

[a] End1 cells or Hec1B cells were infected with MS11 for 3 hours, and the cells were fixed and stained with DAPI, anti-pERM, and an MAb to CD46 ectodomain, Cyt1, or Cyt2, as described in the legend to Fig. 6.

[b] For each microcolony located by DAPI, the presence of pERM was scored. Three independent experiments were performed and plotted as the mean ± the standard deviation. In each experiment, approximately 100 DAPI-positive clusters were scored for the presence of pERM. "*n*" denotes the total number of DAPI-positive microcolonies scored for each pERM signal.

[c] For each cluster of pERM, the presence of CD46 ectodomain, Cyt1, or Cyt2 signal was scored. In these experiments, the presence of microcolonies was confirmed by visualizing DAPI-stained bacteria. Three independent experiments were performed and the results were plotted as the mean ± the standard deviation. In each experiment, approximately 100 pERM-positive clusters were scored for the presence of CD46. "*n*" denotes the total number of pERM clusters scored for each CD46 signal.

may be subject to proteolytic cleavage within cortical plaques. Hakulinen et al. showed that a number of tumor cell lines shed vesicles containing full-length CD46 and secrete soluble forms of CD46 lacking the transmembrane and cytoplasmic tail segments. Furthermore, secretion of soluble CD46 can be blocked by metalloprotease inhibitors (10). If selective cleavage of CD46 Cyt2 isoforms by host and/or bacterial proteases occurs during neisserial infection, the Cyt2 tail may not be retained in the cortical plaque.

A fourth possibility is that antibody detection of Cyt2 within the cortical plaque is blocked by interactions of the tail with other proteins (21, 46) or by phosphorylation (22, 45). The CD46 tail-specific MAbs described in this study were made to nonphosphorylated peptides. The putative core epitope for Cyt2 contains a tyrosine and the Cyt1 epitope contains three threonines and one tyrosine (Fig. 2). Determining the effect of CD46 tail peptide phosphorylation on antibody binding will shed light on the pools of CD46 that are recognized by the tail MAbs.

Why might Cyt1 be clustered in neisseria-induced cortical plaques? Cyt1 and Cyt2 might be recruited differently to cortical plaques. The Cyt1 tail sequence FTSL is essential for interaction with the PDZ domain of Dlg4 (also called PSD-95) (26). PSD-95 (*p*ost*s*ynaptic *d*ensity) family proteins are believed to mediate receptor clustering via interactions with their cytoplasmic tail sequences. Human discs large protein (hDlg), a closely related homolog of Dlg4, can mediate indirect interactions of type 1 membrane proteins with the actin cytoskeleton via its ability to interact with ERM scaffolding proteins (27). Such interactions may help stabilize Cyt1 in cortical plaques that are known to contain actin, ezrin, and pERM (23, 31, 32).

This study has shown that the CD46 ectodomain and Cyt1 and Cyt2 tails can localize to basolateral membranes in polarized T84 cells (Fig. 5). Both Cyt1 and Cyt2 MAbs detected cytoplasmic pools of CD46. Significant apical staining was seen for Cyt2 but not the CD46 ectodomain (while low levels of Cyt1 were detected). A previous study has shown that both Cyt1 and Cyt2 CD46 isoforms localize to basolateral membranes in transfected MDCK cells (28). However, another group demonstrated that CD46 Cyt2 can localize to both apical and basolateral membranes (26). Interestingly, the CD46 Cyt2 constructs used in the two studies were different. Basolateral localization was detected for a BC2 isoform (28), whereas a B2 isoform trafficked to both apical and basolateral membranes (26; S. Russell, personal communication).

Recent studies have called into question whether CD46 is a receptor for type IV pili. CD46 levels in several cell lines do not correlate with the level of binding by piliated *N. gonorrhoeae* or PilC, the major adhesin of type IV pili (19, 44). Our study does not directly address this issue but does show that CD46 can cluster beneath adherent neisseriae. A recent study by Kirchner et al. did not detect CD46 clustering upon *N. gonorrhoeae* infection of epithelial cells (19). The primary difference between our study and theirs was the difference in infection times (3 h versus 90 min). Moreover, we did not centrifuge the inoculum onto the monolayers to initiate infections.

Gill et al. observed enrichment of BC2 and BC1 CD46-GFP fusions underneath adherent neisseriae (8). This study showed that both Cyt1 and Cyt2 isoforms can cluster beneath neisserial microcolonies. Their results contrast with ours in that we did not detect significant levels of Cyt2 clustering (Fig. 6C and Table 1). One possible explanation for this discrepancy in results could be that overexpression of BC2-GFP may have altered the trafficking and/or other properties of the transmembrane protein, preventing it from responding like nontagged CD46 to neisserial infection.

CD46 Cyt2 phosphorylation occurs in response to piliated neisseriae at early time points of infection (22). CD46 downregulation in response to *N. gonorrhoeae* has also been demonstrated to depend on type IV pili (7). The functional consequences of CD46 phosphorylation and downregulation are unclear. Our understanding of the importance of Cyt1 and Cyt2 isoforms to neisserial-

FIG. 6. Immunofluorescence microscopy of CD46 in End1 endocervical cells infected with *N. gonorrhoeae*. End1 cells were infected for 3 h with strain MS11 (3h P.I.) or mock infected with GC broth (3h M.I.), and then fixed and stained with 4′,6′-diamidino-2-phenylindole (DAPI) to visualize DNA from nuclei and bacterial microcolonies (blue signal), a polyclonal antibody to pERM (Cell Signaling Technology) to visualize infection-induced cortical plaques (red signal), and an MAb to CD46 ectodomain (A), Cyt1 (B), or Cyt2 (C). Another view of the location of microcolonies is provided by differential interference contrast (DIC) imaging of the same field of cells from each experiment (leftmost column). Rightmost column: merged CD46, pERM, and DNA images. A single 0.2-μm optical section in the *z* axis plane is shown for each cell. Scale bars, 10 μm.

infection and to general cell function will be facilitated by the tail-specific MAbs generated in this study.

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