

Autophagy Induction by the Pathogen Receptor CD46

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SUMMARY

Autophagy is a highly regulated self-degradative mechanism required at a basal level for intracellular clearance and recycling of cytoplasmic contents. Upon intracellular pathogen invasion, autophagy can be induced as an innate immune mechanism to control infection. Nevertheless, pathogens have developed strategies to avoid or hijack autophagy for their own benefit. The molecular pathways inducing autophagy in response to infection remain poorly documented. We report here that the engagement of CD46, a ubiquitous human surface receptor able to bind several different pathogens, is sufficient to induce autophagy. CD46-Cyt-1, one of the two C-terminal splice variants of CD46, is linked to the autophagosome formation complex VPS34/Beclin1 via its interaction with the scaffold protein GOPC. Measles virus and group A *Streptococcus*, two CD46-binding pathogens, induce autophagy through a CD46-Cyt-1/GOPC pathway. Thus, upon microorganism recognition, a cell surface pathogen receptor can directly trigger autophagy, a critical step to control infection.

INTRODUCTION

Cell invasion by microorganisms is dependent on their ability to bind to cellular receptors that promote their uptake. Among pathogen receptors, members of the regulatory complement activation (RCA) family, including the well-known pathogen receptor CD46, are often used by microorganisms to enter cells (Cattaneo, 2004; Hourcade et al., 2000). Pattern recognition receptors (PRRs) sense pathogens by recognizing pathogen-associated molecular patterns (PAMPs) (Medzhitov, 2007). PRRs of the Toll-like receptor (TLR), nucleotide-binding and oligomerization domain (NOD)-like receptor (NLR), and retinoic acid-inducible gene I (RIG-I)-like receptor (RLR) families signal for innate immune responses leading to proinflammatory cytokine

synthesis, type I interferon (IFN) production, and/or rapid release of IL-1 β (Ishii et al., 2008; Medzhitov, 2007). Another innate defense mechanism is macroautophagy, a highly conserved degradative process shared by all nucleated cells (Xie and Klionsky, 2007). However, the molecular connections between pathogen detection and autophagy are poorly documented.

During macroautophagy (thereafter referred to as autophagy), large portions of the cytoplasm are engulfed into double-membraned autophagosomes that ultimately fuse with lysosomes to form acidic autolysosomes where degradation occurs. Autophagy results from a cascade of reactions orchestrated by autophagy-related genes (*atg*) mostly identified in yeast and for which several orthologs have been characterized in vertebrates (Levine and Klionsky, 2004; Xie and Klionsky, 2007). During autophagy, an isolation membrane is formed upon the activation of the autophagosome formation complex that includes the class III P(I)3-kinase (VPS34) associated with Beclin1 (BECN1) (Furuya et al., 2005; Kihara et al., 2001; Zeng et al., 2006). Then, elongation of the isolation membrane involves two ATG5-dependent ubiquitin-like conjugation systems: the ATG12 conjugation system, leading to the formation of ATG12-ATG5-ATG16 molecular complexes, and the LC3 conjugation system, resulting in the association of the cytosolic microtubule-associated light-chain 3-I (LC3-I) with phosphatidylethanolamine to generate a lipidated LC3-II form. Both conjugation systems bind to the isolation membrane and promote its elongation, leading to the formation of autophagosomes. Finally, autophagosomes mature by fusion with lysosomes, generating degradative single-membraned autolysosomes.

Autophagy contributes to the innate immune response by controlling intracellular pathogens. The physical degradation of intracellular microorganisms is referred to as xenophagy (Levine and Deretic, 2007). Bacterial (Birmingham et al., 2006; Gutierrez et al., 2004; Nakagawa et al., 2004; Py et al., 2007), parasitic (Könen-Waisman and Howard, 2007), and viral (Liang et al., 1998; Liu et al., 2005) infection can be controlled by autophagy in vitro and in vivo (Liang et al., 1998; Shelly et al., 2009; Yano et al., 2008). In mice, *Toxoplasma gondii* clearance involves an ATG5-dependent mechanism; however, it is via an autophagosome-independent process (Zhao et al., 2008). Alternatively, in vitro studies have shown that bacteria (Romano

et al., 2007; Schnaith et al., 2007) and viruses (Jackson et al., 2005) could induce and/or exploit the autophagic machinery to enhance their proliferation/replication, whereas other pathogens evolved strategies to inhibit autophagy (Orvedahl et al., 2007).

Upon intracellular microorganism invasion, autophagy can also facilitate the recognition of cytosolic pathogen components by endosomal TLR in order to promote type I IFN production (Lee et al., 2007), as well as allow for the delivery of peptides derived from cytosolic proteins to major histocompatibility complex (MHC)-II molecules, leading to an adaptive immune response (Paludan et al., 2005; Schmid and Munz, 2007).

Despite the important role of autophagy in immunity against microorganisms, the upstream molecular events leading to the selective induction of this machinery upon initial pathogen detection is poorly identified. TLR engagement was shown to induce autophagy through MyD88- or TRIF-dependent pathways (Delgado et al., 2008; Xu et al., 2007), and interaction between MyD88 and TRIF with BECN1 were reported (Shi and Kehrl, 2008). However, a recent study reports that autophagy induction was not observed upon TLR engagement (Saitoh et al., 2008), and TLR activation was shown to promote autophagy-related protein recruitment to nascent phagosomes but without formation of canonical double-membraned autophagosomes (Sanjuan et al., 2007). In *Drosophila*, the intracellular pathogen sensor PGRP-LE was found to be crucial for the control of *L. monocytogenes* growth through the induction of autophagy, although the molecular mechanism(s) underlying this effect is not known (Yano et al., 2008).

In the present study, we looked for the ability of the ubiquitously expressed human receptor CD46 to promote autophagy. CD46 was an interesting candidate to address for this point because (1) it binds several pathogens such as measles virus (MeV), adenoviruses B and D, human herpes virus 6 (HHV6), *Neisseria* bacteria, and several serotypes of group A streptococcus (GAS) (Cattaneo, 2004); (2) it binds GAS described to be destroyed by autophagy (Nakagawa et al., 2004); (3) it facilitates endosomal TLR9 triggering by DNA adenoviruses (Iacobi-Martinez and Nemerow, 2007); and (4) it promotes measles virus-derived peptide presentation by MHC-II molecules (Gerlier et al., 1994). In this paper, we addressed the questions of whether and how CD46 could induce autophagy and looked at physiological relevance of this process upon pathogen binding.

RESULTS

The Engagement of the Ubiquitous Human Receptor CD46 Increases the Autophagic Flux

To investigate a role of CD46 in the engagement of autophagy, we asked whether antibody-driven CD46 crosslinking could induce autophagosome formation. We have engaged or not CD46 on HeLa-GFP-LC3 cells and measured the formation of GFP-LC3-labeled structures representing LC3-II-containing autophagosomes. Compared to HeLa-GFP-LC3 cells treated with irrelevant IgG (IgG), antibody-driven CD46 crosslinking (α -CD46) increased the number of GFP-LC3 puncta per cell, as did serum starvation (st.), a positive control for autophagy induction (Figure 1A). The reduced expression of the two essential genes for autophagy *ATG5* and *ATG7*, using short interfering (si)RNA (Figure S1 available online), prevented CD46-induced

and starvation-induced autophagy to a similar extent (Figure 1B). Moreover, the prevention of CD46-mediated autophagy in si-*ATG5*-treated cells was reverted by overexpression of *ATG5* (Figures 1C and S2 available online). An electron microscopy analysis revealed an increased number of autophagic vacuoles per cell section in CD46-engaged cells as compared to control cells, which was prevented by 3-methyladenine (3-MA), an inhibitor of autophagosome formation (Figure 1D). Finally, we monitored autophagy following the conversion of LC3-I to LC3-II by western blot. Due to a C-terminal cleavage and association to phosphatidyl ethanolamine, LC3-II has a molecular weight of 16 kD, whereas LC3-I is 18 kD (Klionsky et al., 2008). CD46 engagement led to an increased amount of LC3-II in cell lysates as compared to control cells or to 3-MA-treated CD46-crosslinked cells (Figure 1E).

To determine whether autophagosome detection subsequent to CD46 engagement was the result of an increased autophagic flux or an accumulation of basal autophagosomes due to the inhibition of their degradation, we analyzed autophagy in the presence of lysosomal inhibitors. We used pepstatin A + E64d (P+E) or bafilomycin A1 (BAF), which inhibit lysosomal proteases and acidification of the autolysosomes, respectively. Both inhibitors increased the total number of GFP-LC3 puncta in control cells (Figure 2A), as well as the amount of LC3-II in the total lysate (Figure 2B). Importantly, in the presence of either P+E or BAF, the total number of GFP-LC3 dots was further increased upon CD46 engagement (Figure 2A), as was the increase of the total amount of LC3-II (Figure 2B). Similar results were obtained using chloroquine (CQ), another inhibitor of autolysosome acidification (Figure S3).

To monitor for autolysosome formation, we then used the mRFP-GFP-LC3 probe that allows for the distinction between autophagosomes (GFP⁺RFP⁺ puncta) and autolysosomes (GFP⁻RFP⁺ puncta) due to the quenched signal of the GFP in acidic compartments (Kimura et al., 2009; Vergne et al., 2009). Whereas we found an increased number of total autophagic vacuoles in CD46-crosslinked cells compared with control cells (Figure 2C), an equivalent ratio of number of autophagosomes and autolysosomes was measured in CD46-crosslinked cells and in control or starved cells (Figure 2C). This result indicates that the CD46-induced pathway does not prevent autolysosome formation. Furthermore, CD46-crosslinked cells displayed vacuoles with lysosomal degradative phenotype (Figure S4). Together, these results strongly support that the engagement of CD46 specifically induces de novo autophagosome formation without inhibiting their maturation.

GOPC Connects CD46-Cyt-1 to the Autophagosome Formation Complex VPS34/BECN1

To determine the molecular intermediates involved in CD46-dependent autophagy, we have used CD46 as bait to screen a human cDNA library by yeast two-hybrid. Due to alternative splicing, two groups of CD46 isoforms differing by their C-terminal intracytoplasmic domains, Cyt-1 and Cyt-2 (Figure S5), are coexpressed in all somatic cells (Riley-Vargas et al., 2004). Therefore, Cyt-1 and Cyt-2 were used as bait to screen human cDNA libraries. We have focused on one Cyt-1-interacting protein, GOPC (also known as PIST, CAL, or FIG), that has been previously reported to be associated with an

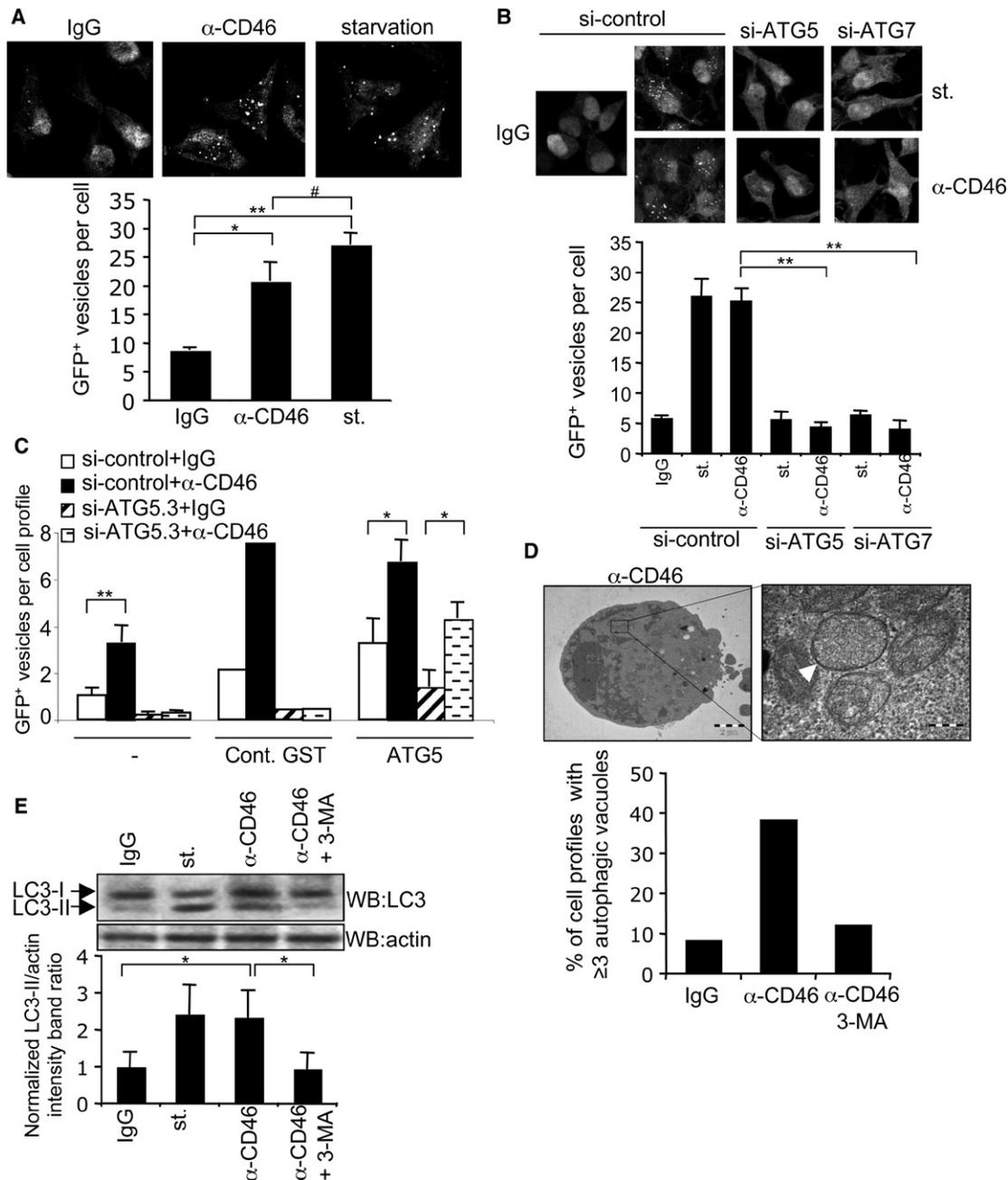


Figure 1. Autophagosome Appearance in Human Cells upon CD46 Engagement

(A) GFP-LC3-HeLa cells were cultured for 4 hr in complete medium either in the presence of coated isotype control antibody (IgG), coated anti-CD46 (α -CD46), or in nutrient-deprived media (starvation/st.). The number of GFP⁺ vesicles per cell was assessed by confocal microscopy. Representative profiles are shown and are accompanied by a graph. Error bars, mean \pm SD of three independent experiments.

(B) GFP-LC3-HeLa cells were pretreated with the indicated si-RNA for 3 days and then incubated and analyzed as in (A). Error bars, mean \pm SD of three independent experiments.

(C) si-Control or si-ATG5-treated GFP-LC3-HeLa cells were untransfected or transfected with an irrelevant GST-encoding vector (one experiment) or with an ATG5-encoding vector and then incubated and analyzed as in (A). Error bars, mean \pm SD of two independent experiments.

(D) HeLa cells were cultured for 3 hr in the presence of irrelevant IgG or α -CD46 \pm 10 mM 3-MA (one experiment). The appearance of autophagic vacuoles was assessed by electron microscopy. A double-membraned autophagosome is enlarged (white arrowhead), and a graph representing the percentage of cell profile with ≥ 3 autophagic vacuoles for each condition are shown.

(E) Starved cells or cells cultured in complete media in the presence of control IgG or α -CD46 \pm 3-MA for 4 hr were lysed, and anti-LC3 western blot was performed. Representative results are shown and are accompanied by a graph representing the intensity of LC3-II/actin bands normalized to the control condition. Error bars, mean \pm SD of three independent experiments.

Student's t test; * $p < 0.05$; ** $p < 0.01$; # $p > 0.05$.

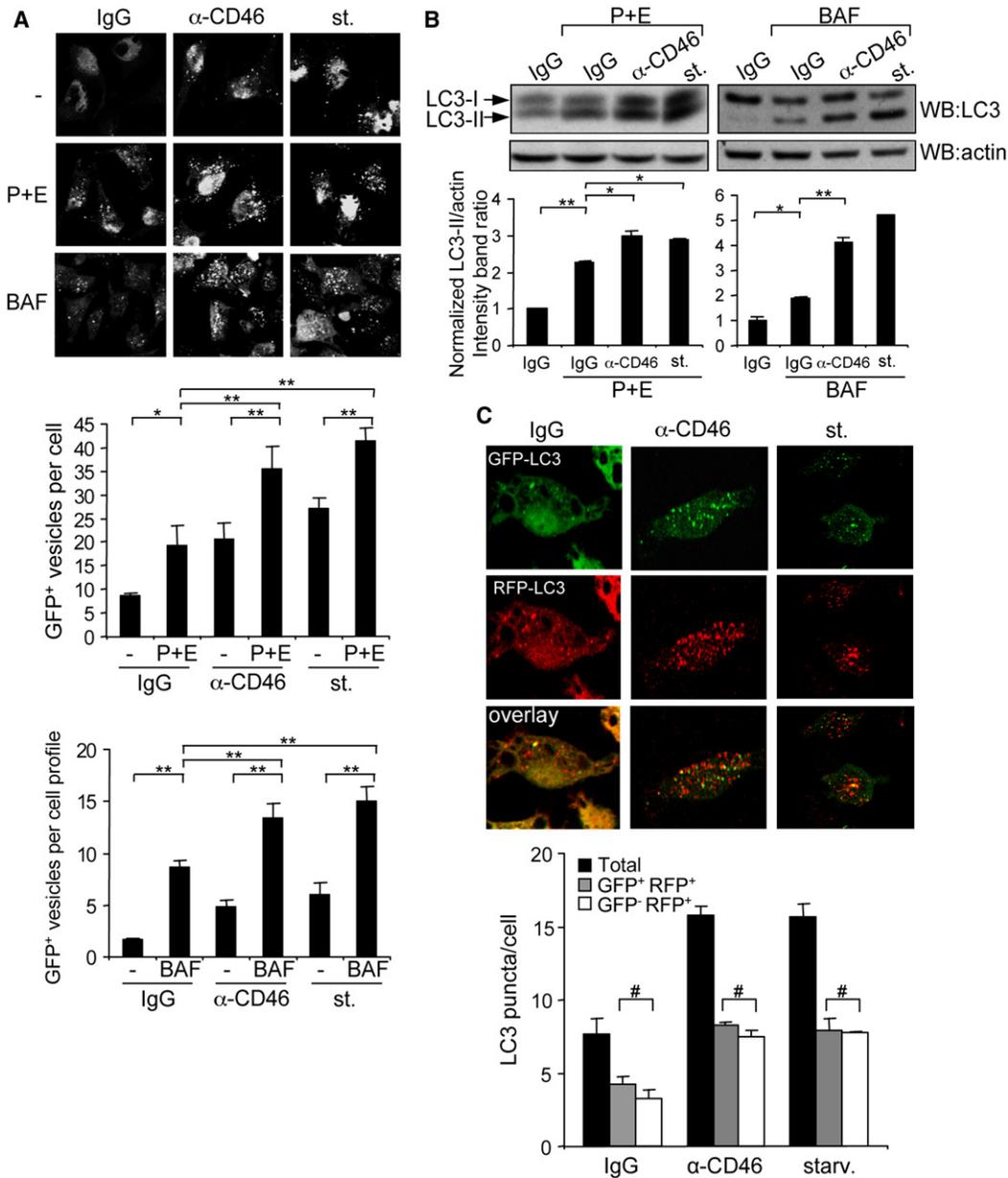


Figure 2. Autophagy Induction upon CD46 Crosslinking

(A) LC3-GFP-HeLa cells were starved (st.) or cultured in the presence of α -CD46 or control IgG for 4 hr alone or in the presence of bafilomycin A1 (BAF) or Pepstatin + E64d (P+E). The number of GFP⁺ vesicles per cell or per cell profile was analyzed by confocal microscopy. Error bars, mean \pm SD of three independent experiments.

(B) HeLa cells were cultured as in (A), and anti-LC3 western blot was performed. Error bars, mean \pm SD of three independent experiments except for one experiment for the st. + BAF condition.

(C) HeLa cells were transfected with mRFP-GFP-LC3. At 24 hr posttransfection, cells were cultured for 4 hr in complete medium either in the presence of coated control IgG, anti-CD46 (α -CD46), or starved (st.). The total number of autophagic vacuoles (GFP⁺RFP⁺ + GFP⁻RFP⁺), autophagosomes (GFP⁺RFP⁺), and autolysosomes (GFP⁻RFP⁺) per cell profiles was numerated. For each experiment, representative profiles of one experiment are accompanied by a graph. Error bars, mean \pm SD of three experiments (IgG and α -CD46) or two experiments (starv.).

Student's t test; *p < 0.05; **p < 0.01; #p > 0.05.

autophagic process (Yue et al., 2002). Twelve clones encoding for human GOPC were found to interact with Cyt-1, but not with Cyt-2 (Figure S5). This specific interaction was confirmed by a yeast two-hybrid matrix using a full-length clone of GOPC (isoform b) as a prey (Figure 3A). The specificity of the interaction

was further confirmed in HEK293T human cells by affinity copurification of myc-tagged GOPC with GST-tagged Cyt-1, but not with GST alone or GST-Cyt-2 (Figure 3B).

GOPC is a scaffold protein that contains two coiled-coil domains (CC) and a PDZ domain (Figure 3C). To determine

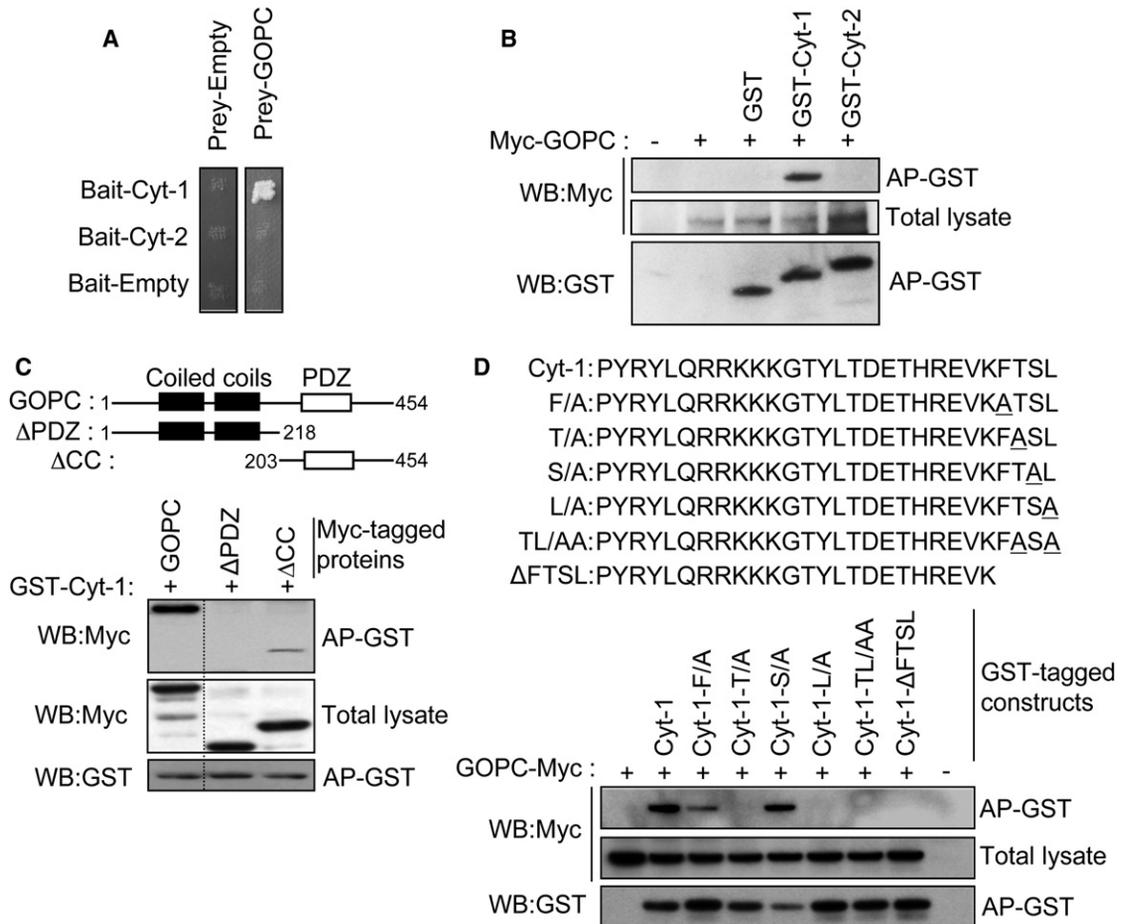


Figure 3. Interaction of GOPC with CD46-Cyt-1

(A) Yeast two-hybrid assay of Cyt-1 or Cyt-2 used as bait and GOPC used as prey on 3AT selective medium (one experiment representative of two). (B) Anti-Myc and anti-GST western blots (WB) on coaffinity-purified (AP) complexes from HEK293T cells transfected with the indicated construct-encoding plasmids (one experiment representative of three). (C) Schematic representation of GOPC and GOPC-ΔPDZ (ΔPDZ) and GOPC-ΔCC (ΔCC) mutant constructs used in this study. HEK293T cells were transfected with the indicated constructs, and co-AP and western blots were performed as in (B) (one experiment representative of three). (D) Mutants of Cyt-1 C-terminal sequence used to screen the interaction with GOPC are shown. A Myc-tagged GOPC plasmid was transfected alone or in combination with the indicated plasmid encoding GST-tagged Cyt-1 mutant constructs. Co-AP and western blots were performed as in (B) (one experiment representative of three).

whether one of these domains was involved in the interaction with Cyt-1, we generated mutant constructs of GOPC either deleted for the CC domains (ΔCC) or for the PDZ domain (ΔPDZ) (Figure 3C). In coaffinity purification experiments, the GOPC-ΔCC construct could still interact with Cyt-1, whereas the GOPC-ΔPDZ construct lost this ability, indicating that the PDZ domain of GOPC is involved in the interaction with Cyt-1. The GOPC PDZ domain is a class I PDZ domain predicted to interact with the consensus X [T/S] X [L/I/V] C-terminal sequences of partner proteins (Songyang et al., 1997). Such a consensus motif was found in Cyt-1 (FTSL), but not in Cyt-2 (Figure S5). Of interest, deletion of the last four C-terminal residues of Cyt-1 (ΔFTSL) abolished Cyt-1 interaction with GOPC (Figure 3D). Point mutations for an A on the critical T and/or L residues for PDZ binding (TL/AA, T/A, and L/A constructs) (Figure 3D) had a similar disruptive effect, whereas the mutation of either of

the dispensable F or S residues (F/A or S/A constructs) did not prevent the GOPC/Cyt-1 interaction (Figure 3D). Thus, GOPC is a new intracellular partner of CD46 that can interact with the Cyt-1 C-terminal tail via its class I PDZ domain.

A murine neuron-specific isoform of GOPC, nPIST, was shown to interact with BECN1 (Yue et al., 2002). However, GOPC/ BECN1 interaction in human cells has not been reported. Coaffinity purification experiments showed that the human ubiquitously expressed isoform b of GOPC can interact with human BECN1 (Figure 4A). Moreover, the GOPC-ΔCC mutant construct lost the ability to bind to BECN1, whereas the GOPC-ΔPDZ construct did not (Figure 3C and 4A). Thus, human GOPC can bind CD46-Cyt-1 via its PDZ domain and BECN1 via its CC domains.

In transfected cells, both Myc-BECN1 and endogenous VPS34 can be copurified with GST-GOPC, but not with GST alone (Figure 4B), suggesting that GOPC can associate with

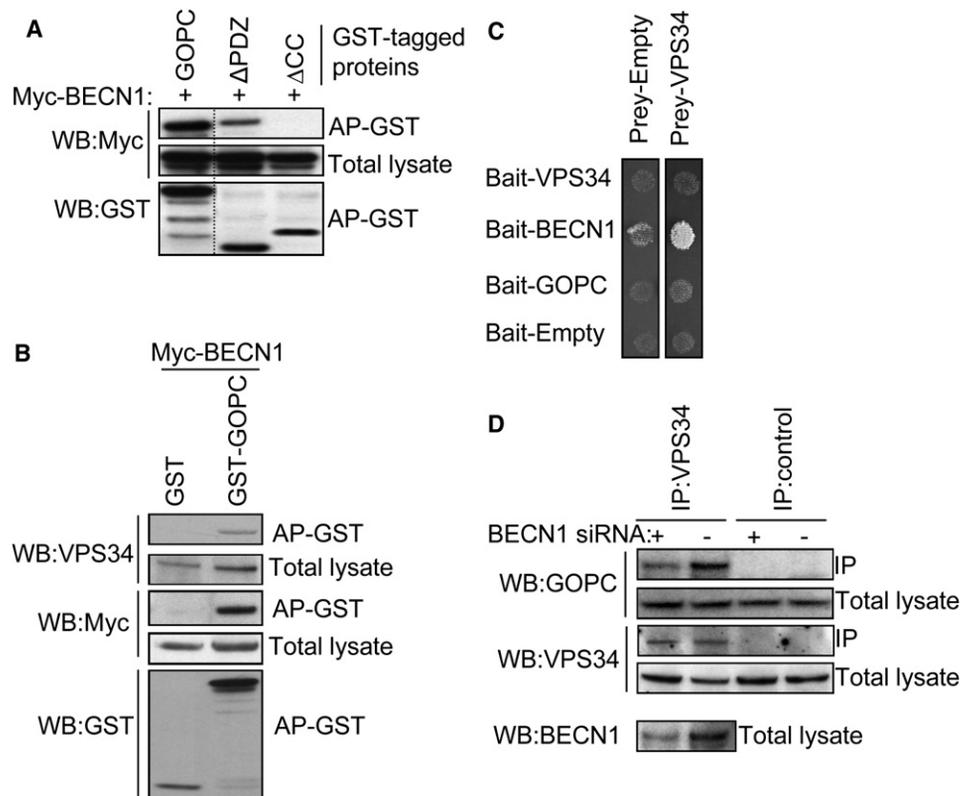


Figure 4. Interaction of GOPC with the Autophagosome Formation Complex BECN1/VPS34

(A) Cells were cotransfected with Myc-BECN1 and GST-tagged GOPC, GOPC- Δ PDZ, or GOPC- Δ CC protein-encoding plasmids, and co-AP experiments were performed (one experiment representative of three).

(B) HEK293T cells were cotransfected with GST and Myc-BECN1 or GST-GOPC and Myc-BECN1, and a co-AP was performed. Anti-VPS34, anti-Myc, and anti-GST were used for western blot (one experiment representative of three).

(C) VPS34, BECN1, or GOPC were used as baits, and VPS34 was used as a prey (one experiment representative of two).

(D) HEK293T cells were either untreated or treated with *BECN1*-targeting siRNA. Coimmunoprecipitation experiments were performed using anti-VPS34 (IP: VPS34) or irrelevant (IP: control) antibodies. Endogenous GOPC, BECN1, and VPS34 were revealed by western blot (one experiment representative of two).

the autophagosome formation complex VPS34/BECN1. To determine whether GOPC binds VPS34 independently of BECN1, we tested whether GOPC could directly interact with VPS34 in a yeast two-hybrid assay. No interaction was detected between VPS34 and GOPC, whereas VPS34/BECN1 interaction was detected as expected (Figure 4C). We reasoned that, if BECN1 can bridge GOPC and VPS34, then less GOPC should be associated to VPS34 in cells in which BECN1 expression is downregulated. Indeed, in HEK293T cells silenced for the expression of BECN1, less endogenous GOPC can be immunoprecipitated with VPS34 (Figure 4D).

The Engagement of CD46-Cyt-1 Induces Autophagy through a Direct Pathway

Molecular analysis suggested a role for the intracytoplasmic tail of CD46-Cyt-1 and for GOPC in CD46-mediated autophagy. We tested the involvement of the CD46-Cyt-1/GOPC/BECN1 pathway in this process using siRNA. Real-time RT-PCR analysis revealed that HeLa cells express both CD46-Cyt-1 and CD46-Cyt-2 isoforms at a ratio close to that of human primary cells (Figure 5A, compared HeLa to peripheral blood lymphocytes [PBLs]). If the genomic organization of *CD46* allows for the

design of *Cyt-1*-specific siRNA, it is not possible to specifically shut down the expression of *Cyt-2*. si-*Cyt-1* treatment of HeLa cells downregulated the expression of the *CD46-Cyt-1* mRNA, without modifying the expression of *CD46-Cyt-2* mRNA (Figure 5B). Moreover, total surface expression of CD46 was only slightly affected in si-*Cyt-1*-treated cells (Figure S6). However, the engagement of CD46 on si-*Cyt-1*-treated cells did not induce autophagy (Figure 5C). Thus, in contrast to CD46-Cyt-1, CD46-Cyt-2 engagement does not seem to trigger autophagy. Importantly, starvation-induced autophagy remained possible in si-*Cyt-1*-treated cells, indicating that these cells do not suffer from a general alteration of the autophagic process (Figure 5C). Furthermore, CD46-induced autophagy was abolished when GOPC expression was downregulated (Figure S7), whereas starvation-induced autophagy remained unchanged (Figure 5C). On the contrary, downregulation of BECN1 expression (Figure S7) abolished both CD46 and starvation-induced autophagy, showing that BECN1 is a crucial molecule for both pathways (Figure 5C). These results suggest that upstream events are not shared between CD46-induced autophagy and starvation-induced autophagy, given that CD46-Cyt-1-mediated autophagy is strictly dependent on GOPC.

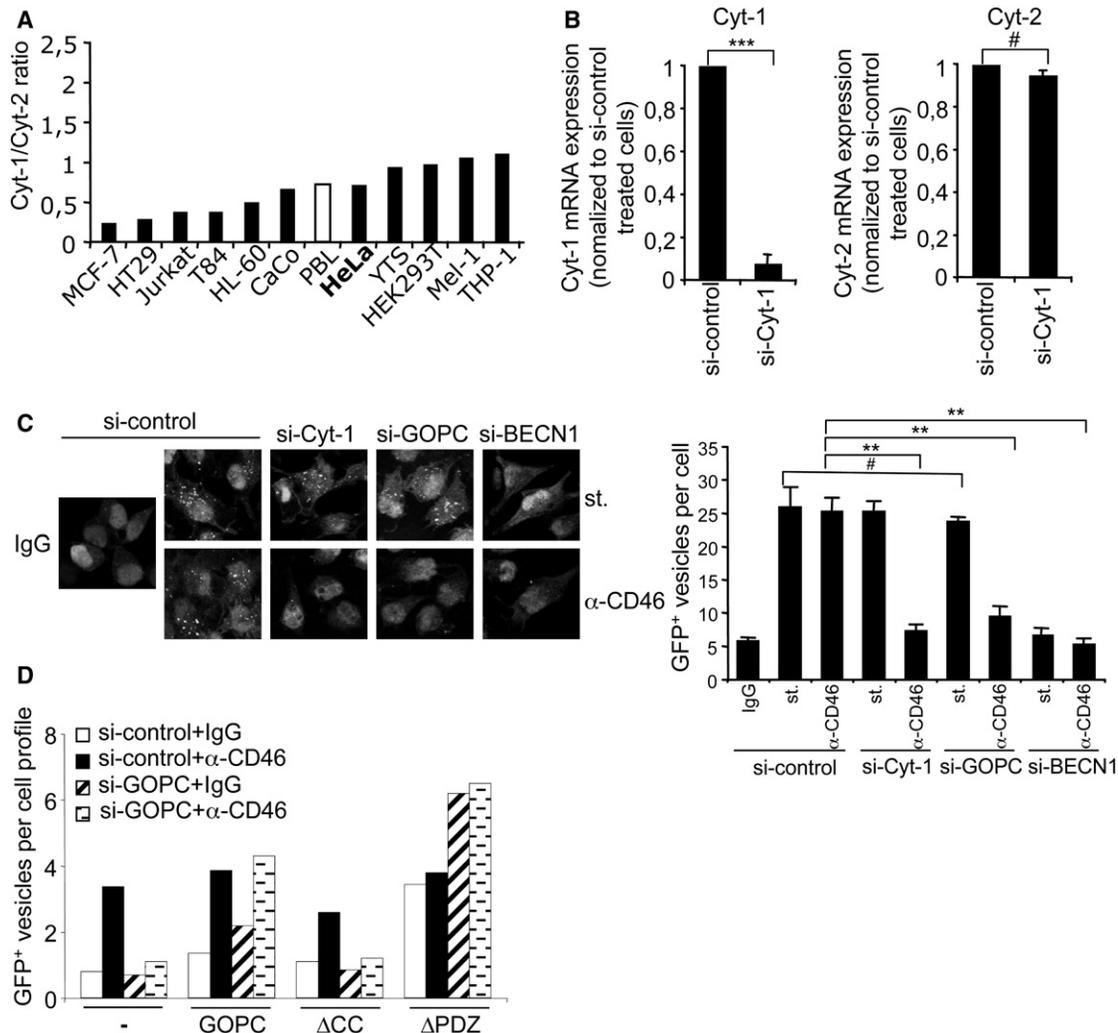


Figure 5. Autophagy Induction upon CD46 Crosslinking Is Dependent on the Cyt-1 Isoform and GOPC

(A) *CD46-Cyt-1* and *CD46-Cyt-2* mRNA levels were assessed by RT-qPCR in the indicated cell lines (black histograms) and in freshly isolated human PBL (white histogram) (one experiment).

(B) *CD46-Cyt-1* and *CD46-Cyt-2* mRNA expression levels in si-Cyt-1-treated HeLa cells normalized to si-control-treated cells. Error bars, mean \pm SD of three experiments.

(C) GFP-LC3-HeLa cells were treated with the indicated siRNA. Autophagy in starved HeLa cells (st.) or cells cultured in the presence of α -CD46 was analyzed as in Figure 1. Representative labeled cells and a graph are shown. Error bars, mean \pm SD of three independent experiments.

(D) si-Control or si-GOPC-treated GFP-LC3-HeLa cells were untransfected or transfected with a GOPC or GOPC- Δ PDZ (Δ PDZ) or GOPC- Δ CC (Δ CC) mutant constructs. Cells were then either incubated with an irrelevant IgG or crosslinked for CD46, and autophagy was monitored as in Figure 1 (one experiment representative of two).

Student's t test; **p < 0.01; #p > 0.05).

We further found that the prevention of CD46-induced autophagy in si-GOPC-treated cells was reverted upon full-length GOPC overexpression, but not upon overexpression of GOPC- Δ CC or GOPC- Δ PDZ constructs (Figure 5D). Moreover, the overexpression of the GOPC- Δ PDZ construct promoted autophagosome accumulation (as reported elsewhere [Yue et al., 2002]) but competed with endogenous GOPC for CD46-dependent induction of autophagy. On the contrary, the GOPC- Δ CC construct was not efficient to compete with endogenous GOPC. A possible explanation could be the titration of the GOPC- Δ CC construct by other proteins able to bind a PDZ domain (Figure 5D). Together, these results indicate that

GOPC is crucial in the induction of autophagy upon CD46 engagement.

Measles Virus Induces Autophagy through a CD46-Cyt-1/GOPC Pathway

Our laboratory identified CD46 as the receptor for laboratory and vaccinal MeV strains (Naniche et al., 1993). To determine whether the engagement of CD46 by a natural ligand could also induce autophagy, we infected cells with the laboratory Edmonston strain of MeV. Early after infection (1.5 hr postinfection), we found that the amount of LC3-II in the total cell lysate was increased compared to uninfected cells (Figure 6A). The

number of GFP-LC3⁺ autophagosomes per cell profile was also strongly increased in MeV-infected as compared to uninfected cells (Figure 6B), which were abolished in cells pretreated with si-*ATG5* or si-*BECN1*. Moreover, MeV induced de novo formation of autophagosomes, as revealed by experiments using BAF, P+E, or CQ treatments (Figures 6C, 6D, and S8), and such autophagosomes fully matured into autolysosomes, as shown by experiments using the mRFP-GFP-LC3 probe (Figure 6E). Finally, the downregulation of either CD46-Cyt-1 or GOPC expression with siRNA prevented MeV-induced autophagy, as analyzed by tracking GFP-LC3⁺ dots (Figure 6B). Thus, these results show that MeV infection induces autophagy upon a CD46-Cyt-1/GOPC pathway.

The CD46-Cyt-1/GOPC Pathway Sensitizes Cells for Autophagic Degradation of GAS

CD46 is also a receptor for several serotypes of GAS, a bacteria destroyed by autophagy (Cattaneo, 2004; Nakagawa et al., 2004). To investigate the role of the CD46-Cyt-1/GOPC pathway in GAS targeting to autophagy, we incubated HeLa-GFP-LC3 cells with CD46-binding emm6⁺ GAS (Giannakis et al., 2002) and analyzed colocalization of the bacteria with GFP-LC3 puncta. Whereas almost 80% emm6⁺ GAS colocalized with GFP-LC3 vesicles in infected control cells, this percentage dropped to less than 40% in *ATG5* or *BECN1* siRNA-treated cells (Figure 7A) in accordance with a previous report (Nakagawa et al., 2004). The reduced expression of GOPC did not impede the GAS intracellular invasion, whereas the reduced expression of Cyt-1 decreased by 60% GAS invasion (Figure S9). However, the reduced expression of either GOPC or Cyt-1 strongly compromised the intracellular GAS targeting to autophagosomes, with less than 30% of GAS colocalizing with GFP-LC3 puncta in *GOPC* or *Cyt-1* siRNA-treated cells (Figures 7A and S10). To determine the impact of the CD46-Cyt-1/GOPC pathway in GAS killing, cells were infected for 1 hr, and viable intracellular bacteria were scored by counting colony-forming units. In accordance with published observations (Nakagawa et al., 2004), GAS were spared from destruction in cells treated either with *ATG5*- or *BECN1*-targeting siRNA (Figure 7B). Of interest, the reduced expression of either CD46-Cyt-1 or GOPC significantly reduced the cell's ability to destroy intracellular GAS as well (Figure 7B).

GAS are efficiently degraded by autophagy in murine cells, although these cells do not express CD46. Moreover, GAS autophagic sequestration in these cells was reported to follow cytosolic delivery of GAS from endosomes (Nakagawa et al., 2004). These points raised the possibility that other intracellular molecules might sense GAS and trigger autophagy. In contrast to 1 hr postinfection, after 3 hr of infection, intracellular GAS were spared from destruction in cells with reduced expression of *ATG5*, but not in cells with reduced expression of either CD46-Cyt-1 or GOPC (Figure 7C). Moreover, a GAS serotype that does not bind CD46 (emm49⁺ serotype) (Darmstadt et al., 2000) was degraded inside of invaded cells but with a significant delay as compared with CD46-binding GAS serotype (emm6) (Figure 7D). Thus, these results indicate that, in human cells, CD46-Cyt-1/GOPC-dependent early autophagosome formation signals GAS invasion to the host cell.

DISCUSSION

CD46 and Autophagy

CD46 is a type I glycoprotein expressed by all nucleated human cells. Due to alternative splicing, 12 isoforms of CD46 can be expressed, which mostly differ at the level of their extracellular membrane-proximal domains (Riley-Vargas et al., 2004). Two different cytoplasmic tails are found in CD46 isoforms, Cyt-1 and Cyt-2, which do not show any sequence similarity and are coexpressed in human somatic cells. Both CD46-Cyt-1 and CD46-Cyt-2 isoforms follow independent but identical internalization pathways that are dependent on the degree of ligand crosslinking (Crimeen-Irwin et al., 2003). CD46 is constitutively internalized in clathrin-coated pits, transits to multivesicular bodies (MVB), and is recycled to the cell surface. Of interest, ligand-induced aggregation of CD46 leads to the extension of membrane ruffles, which forms macropinosomes-like vesicles of about 0.5–1 μm in diameter and targets CD46 for intracellular degradation. It was proposed that the ligand-induced pathway may facilitate pathogen entry. We report here that only CD46-Cyt-1 crosslinking induces autophagy, indicating that the issue of the macropinosomes-like vesicles may differ according to the cytoplasmic isoform engaged.

The C-terminal tetrapeptide FTSL of CD46-Cyt-1 interacts specifically with the class I PDZ domain of GOPC (Figure 3). The PDZ domain is one of the most common protein-protein interaction domains in humans and is a key factor in the signal transmission within multiple cellular pathways (Craven and Brecht, 1998; Wang et al., 2008). Moreover, the relative binding affinities of PDZ-containing partners for receptors may influence biological responses (Cushing et al., 2008). The FTSL domain of Cyt-1 binds at least to another class I PDZ domain expressed by the scaffold protein DLG4. DLG4 is responsible for its copolarized expression with CD46-Cyt-1 in epithelial cells (Ludford-Menting et al., 2002). Thus, GOPC might substitute for another PDZ partner upon aggregation-induced CD46-Cyt-1 internalization. The reduced expression of GOPC did not have an impact on GAS intracellular invasion, whereas the reduced expression of Cyt-1 did, suggesting that GOPC is not involved in the internalization process of CD46-Cyt-1. The CD46-Cyt-1/GOPC interaction could confer differential structural and/or location properties to the scaffold protein, allowing an improved stabilization of basal VPS34/BECN1 association/activation or facilitating new complex formation leading to autophagy. In support of this hypothesis, whereas the overexpression of full-length GOPC did not increase basal autophagy, overexpression of GOPC- Δ PDZ construct did. GOPC is not involved in starvation- and rapamycin-induced autophagy (Figure 5 and data not shown), suggesting that its involvement in autophagy is dependent on specific upstream signals.

First identified as a Golgi-associated protein, GOPC was described to be involved in intracellular trafficking and to promote receptor recycling and degradation (Charest et al., 2001; Cheng et al., 2004). Of interest, the association of a neuronal isoform of GOPC, nPIST, to a constitutively active $\delta 2$ glutamate receptor (GluR $\delta 2$) in Purkinje cells of pathological lurcher mice was shown to synergize autophagy with BECN1, possibly responsible for neurodegeneration (Yue et al., 2002). Strikingly, the cystic fibrosis transmembrane conductance regulator

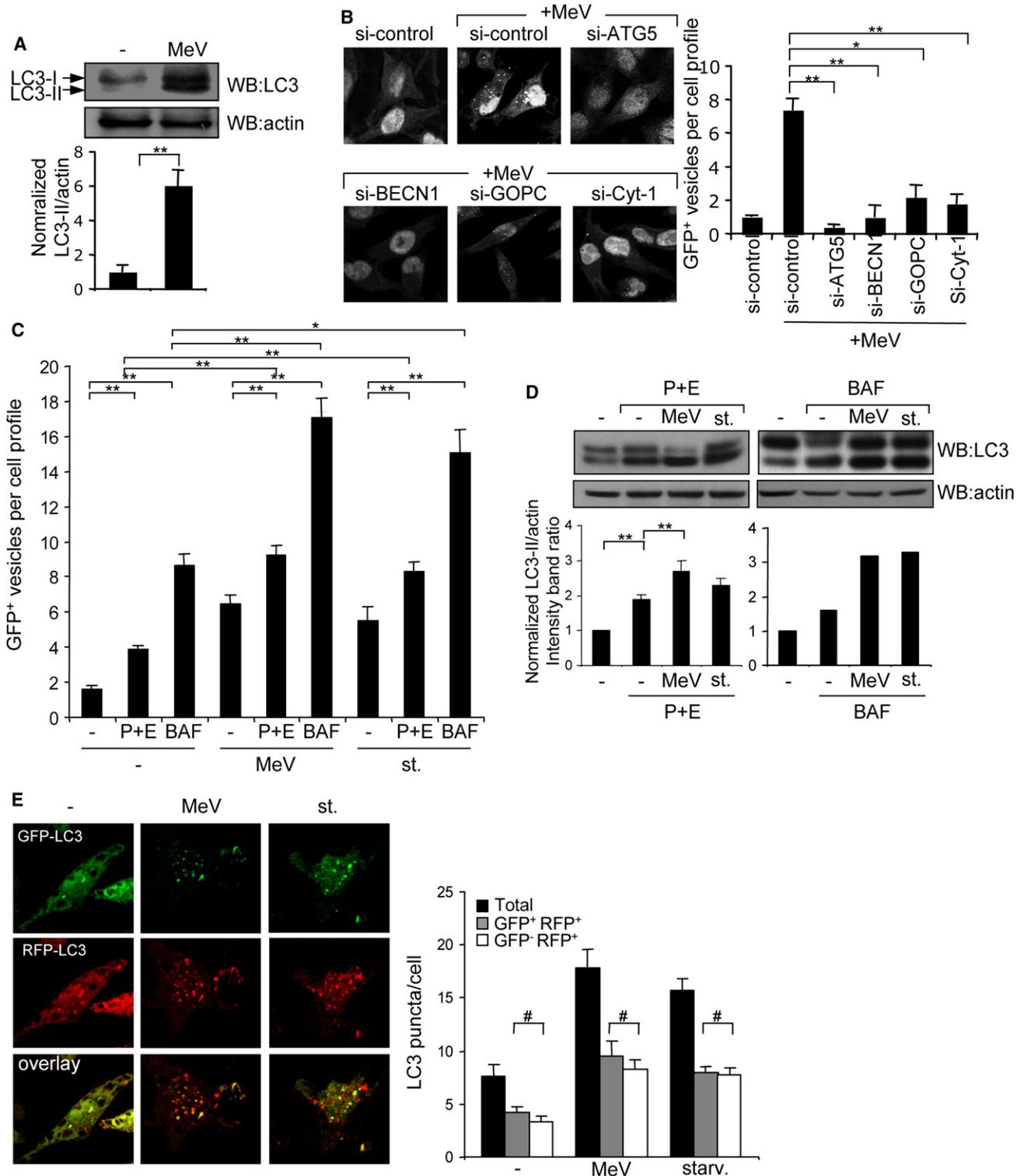


Figure 6. CD46-Cyt-1/GOPC-Dependent Autophagy Induction by Measles Virus

(A) HeLa cells were uninfected (-) or infected by purified MeV Edmonston strain (MeV) for 1.5 hr and lysed, and an anti-LC3 western blot was performed. Representative results are accompanied by a graph as in Figure 1. Error bars, mean \pm SD of three independent experiments.

(B) GFP-LC3-HeLa cells treated with the indicated siRNA were infected with MeV for 1.5 hr, and the number of GFP⁺ vesicles per cell profile was assessed by confocal microscopy. Representative results and a graph are shown. Error bars, mean \pm SD of three independent experiments.

(C and D) Cells were treated as in (B) but in the presence of BAF or P+E. A starvation condition is used as a positive control for autophagy. Autophagy was monitored by confocal microscopy (error bars, mean \pm SD of three independent experiments) (C) or by western blot (error bars, mean \pm SD of three independent experiments for P+E; one experiment in the presence of BAF) (D) as in Figure 1.

(CFTR) is subjected to lysosomal degradation when bound to GOPC, suggesting that other cellular receptors might be connected to autophagy via GOPC (Cheng et al., 2004).

CD46-Mediated Autophagy and Immune Responses

CD46 is a well-known pathogen receptor that binds to vaccine strains of MeV, several serotypes of GAS, HHV6, adenoviruses B and D, and *Neisseria* bacteria (Cattaneo, 2004). CD46 is known to impair T cell effector function, rendering this receptor attractive for pathogens (Kemper and Atkinson, 2007; Meiffren et al., 2006). However, we report here that CD46 could control early pathogen infection by xenophagy, as we show that the CD46-Cyt-1/GOPC pathway participates in the targeting of GAS to an autophagic degradation. This pathway induces the formation of an early and readily available pool of autophagosomes, which can efficiently sequester and degrade GAS without any further delay. Our data also indicate that other intracellular sensors can take over the CD46-Cyt-1/GOPC pathway for GAS delivery to autophagosomes, suggesting that several pathogen molecular detectors may be available to initiate autophagy in order to optimize cellular innate defense against invading pathogens.

CD46 is a regulatory complement activation (RCA) family member that protects cells from complement autolysis by binding C3b and C4b complement activation fragments to allow their degradation by factor I. Thus, CD46 might also bind C3b/C4b-opsonized bacteria (Riley-Vargas et al., 2004). Of interest, uropathogenic C3b-opsonized *Escherichia coli* are degraded inside of epithelial cells through a CD46-dependent mechanism that could be autophagy (Li et al., 2006). Whether the CD46-Cyt-1/GOPC-dependent pathway induces autophagy in the context of other pathogens has to be specifically addressed.

Some pathogens developed strategies to avoid autophagic degradation (Kirkegaard et al., 2004; Levine and Deretic, 2007). Whereas two C-terminal splice variants of CD46 are expressed in most cells, only CD46-Cyt-1 induces autophagy. Of interest, an altered regulation of the Cyt-1/Cyt-2 ratio in patients with multiple sclerosis was reported, which correlated with altered lymphocyte differentiation upon CD46 engagement (Astier et al., 2006). An attractive hypothesis to investigate is whether pathophysiological signals (for instance, pathogens themselves) could influence autophagy by altering CD46 splice regulation.

Autophagy favors the delivery of antigenic peptides on MHC-II and promotes adaptive immune response initiation (Schmid and Munz, 2007). Our previous studies argued for a role of CD46 in MeV-derived peptides' presentation on MHC-II molecules through a mechanism that is sensitive to lysosomal acidification inhibition (Gerlier et al., 1994). It has to be determined whether MeV degradation mediated by autophagy could favor MHC-II peptide presentation, which might explain the protective immunity of MeV based-vaccines versus the virulence of clinical strains of MeV that inefficiently bind to CD46 (Manchester et al., 2000; Schneider et al., 2002).

Despite the central role of autophagy in innate immune control of microorganisms, the molecular events leading to the selective induction of this machinery upon pathogen detection are still largely unknown. TLR3, TLR4, and TLR7 in mammals, as well as PGRP-LE in *Drosophila*, were reported to induce autophagy upon ligand binding. However, the molecular connections between these receptors and autophagy are unknown and may require transcriptional events (Delgado et al., 2008; Xu et al., 2007; Yano et al., 2008). We report here that the pathogen receptor CD46 can be physically connected to the autophagic machinery via a unique molecular intermediate. This property confers to this receptor the potency to sense pathogens and to initiate an innate immune response.

EXPERIMENTAL PROCEDURES

Cells, Antibodies, and Reagents

HeLa cells were maintained in RPMI 1640 and HEK293T cells in DMEM (Invitrogen, Cergy Pontoise, France) + 0.5 mg/ml gentamycin, 2 mM L-glutamine, and 10% fetal calf serum. Cells cultured in 6-well plates were transfected with Lipofectamine 2000 reagent (Invitrogen) according to manufacturer's instructions. HeLa cells were transfected with pGFP-LC3 plasmid, and a stable HeLa-GFP-LC3 cell line clone was established by limited dilution in 0.05 mM genitcin-selective medium. Antibody used were: anti-GST (clone B-14) and anti-BECN1 (clone E-8) (mouse monoclonal, Santa Cruz Biotechnology, CA); anti-c-Myc, anti-ATG5, and anti-MAP-LC3B (rabbit polyclonal, Sigma-Aldrich, Saint Quentin Fallavier, France); anti-VPS34 (rabbit polyclonal, Zymed/Invitrogen); anti-CD46 (mouse monoclonal, clone 20.6 produced in the lab); anti-GOPC (GOPC-GST rabbit anti-serum, produced by the lab); and MOPC-21 (mouse monoclonal irrelevant IgG, BD Biosciences, Le-Pont-De-Claix, France). Pharmacological inhibitors used were 3-methyl-adenine (10 mM), bafilomycin A1 (100 nM), chloroquine (50 μ M), or Pepstatin + E64d (10 μ M) and were added 15 min prior to cell treatment and kept for the entire duration of the experiments.

Molecular Cloning, Coaffinity Purification, and Coimmunoprecipitation

Cyt-1 and Cyt-2 were amplified from plasmids pVZ-5.1 encoding isoform CD46BC-Cyt-1 and pHMGC-CD46 encoding isoform CD46C-Cyt-2, respectively. Human monocyte cDNA served as template to clone GOPC (isoform b) and *BECN1*. GST- or Myc-tagged constructs were generated with the Gateway system (Invitrogen). Expression vectors were transfected in HEK293T cells, and after 24 hr of culture, cells were lysed in ice-cold buffer (1% NP-40, 40 mM Tris [pH 8], and 200 mM NaCl plus protease inhibitor complete Mini EDTA-free [Roche Diagnostics GmbH, Mannheim Germany]) before coaffinity purification or coimmunoprecipitation. For coaffinity purification, 30 μ l of 50% slurry glutathione Sepharose 4B beads (GE healthcare, Saint Cyr au Mont d'Or, France) were added to 300 μ g of total lysate proteins in a final volume of 700 μ l, incubated for 1.5 hr at 4°C, and washed five times with 500 μ l of lysis buffer before western blotting. For coimmunoprecipitation, 1 μ g/ml of anti-VPS34 or irrelevant IgG was added to 300 μ g of total lysate proteins in a final volume of 700 μ l and incubated for 15 min at 4°C before adding 30 μ l of 50% slurry protein G Sepharose (GE healthcare). The mixture was then incubated for 1.25 hr at 4°C and washed as described for coaffinity purification.

siRNA

Smartpool siRNA targeting *ATG5*, *ATG7*, *BECN1*, *GOPC*, control siRNA, and Cyt-1-specific siRNA (AUACCUAACUGAUGAGACCUU) were all from Dharmacon (Perbio, Brebières, France). In some experiments, individual Stealth *ATG5* RNAi or control were used (Invitrogen). HeLa cells (50×10^3) were

(E) At 24 hr posttransfection with mRFP-GFP-LC3, HeLa cells were either untreated, infected with MeV for 1.5 hr, or starved. Maturation of autophagosomes was estimated as in Figure 2C. Representative profiles of one experiment are accompanied by a graph. Error bars, mean \pm SD of three experiments (– and MeV) or two experiments (starv.).

Student's t test; * $p < 0.05$; ** $p < 0.01$; # $p > 0.05$.

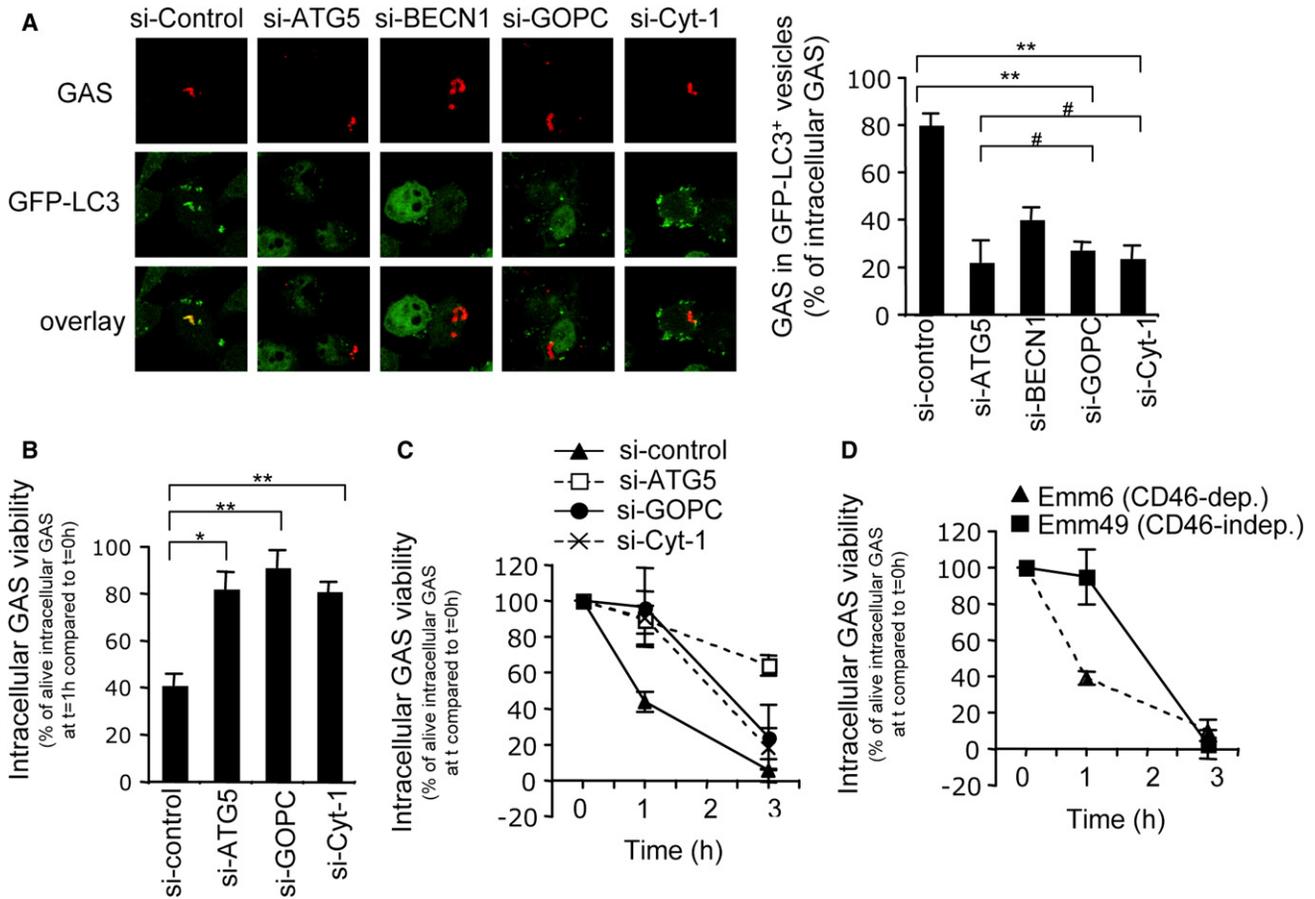


Figure 7. Cell Sensitivity to Group A *Streptococcus* Infection upon CD46-Induced Autophagy

(A) GFP-LC3-HeLa cells treated with the indicated siRNA were infected for 2 hr with PKH26-labeled emm6+ GAS (red) and then fixed for confocal microscopy analysis. Representative infected cells are shown, and the percentage of intracellular labeled GAS colocalized with GFP-LC3 vesicles (yellow) is represented on a graph.

(B) Cells treated with the indicated siRNA were infected for 1 hr with emm6+ GAS strain. Cells were then washed, and extracellular GAS were killed by ampicillin treatment (t = 0 hr). Cells were then cultured for 1 additional hr (t = 1 hr). Intracellular GAS viability at t = 1 hr/t = 0 hr is presented as the ratio of alive intracellular GAS at t = 1 hr/t = 0 hr.

(C) Cells were treated as in (B), and a kinetic is presented.

(D) HeLa cells were infected with emm6+ or emm49+ GAS strains, and intracellular GAS viability was measured as in (B) and (C).

Error bars, mean \pm SD of three independent experiments. Student's t test; *p < 0.05; **p < 0.01; #p > 0.05.

cultured in 6-well plates for 1 day in OptiMEM (Invitrogen) 10% FCS before transfection of 30 nM siRNA with Lipofectamine RNAiMAX (Invitrogen) according to manufacturer's instructions. mRNA expression level was assessed by quantitative RT-PCR after 2 days (Meiffren et al., 2006) or western blotting after 3 days before using cells for experiments.

Yeast Two-Hybrid

Screens were performed as described elsewhere (Walhout and Vidal, 2001).

Autophagy Induction

Plates were precoated with 15 μ g/ml 20.6 or MOPC-21 antibodies (both IgG1 isotype) in PBS for 3 hr at 37°C, washed twice with PBS, and cells cultured in precoated plates for 2–4 hr in complete RPMI medium. Starvation for the same period of time was used as a positive control for autophagy. Cells were cultured in supplemented RPMI medium without FCS for 2–4 hr at 37°C. In some experiments, 24 hr siRNA-treated HeLa-GFP-LC3 cells were transfected with Flag-ATG5, GOPC, GOPC- Δ CC, or GOPC- Δ PDZ protein-expressing vectors. At 48 hr posttransfection, CD46 was crosslinked and autophagy monitored by confocal microscopy.

Confocal

HeLa-GFP-LC3 cells were fixed with 4% paraformaldehyde and then analyzed using an Axiocvert 100M microscope (Zeiss, Göttingen, Germany) equipped with the LSM 510 system (Zeiss) and observed with a magnification of 63 \times (oil immersion). The number of GFP+ vesicles per cell was numerated from 12–15 Z sections collected at 0.5 μ m intervals from individual cells. The number of GFP+ vesicles per cell profile was numerated from one single plan section per cell. In each case, the number of GFP+ vesicles was numerated from 100 to 200 cells for each experiment. For some experiments, cells were cultured with dyes, LysoTracker (Invitrogen) (1 μ M) and DQRed (10 μ g/ml), for 15 min at 37°C in culture medium, washed four times in PBS, and fixed with 4% paraformaldehyde. The mRFP-GFP-LC3 construct was transiently expressed in HeLa cells 24 hr prior to starvation, CD46 crosslinking, or MeV infection. The total number of autophagic vacuoles (total: GFP+RFP+ + GFP-RFP+), autophagosomes (GFP+RFP+), and autolysosomes (GFP-RFP+) were determined from at least 25 cells per experiment.

Electron Microscopy

Cells were fixed in 0.1 M sodium-cacodylate and 2% glutaraldehyde buffer at RT for 40 min and washed three times in sodium cacodylate 0.2 M for 10 min at

RT. Analyses were performed at the Centre Technologique des Microstructures de la Doua (Villeurbanne, France).

MeV Infection

HeLa-GFP-LC3 cells were infected with 1 particle-forming unit (pfu)/cell of Vero cell-derived MeV Edmonston, and at 1.5 hr postinfection, autophagy was monitored by confocal analysis.

GAS Strains, Labeling, Infection, and Viability Assay

Clinical GAS strains used were of emm6 and emm49 serotype (kind gifts of Pr. POYART, French National Reference Center for Streptococci, Paris, France). Mid-log phase grown bacteria were harvested and washed twice in PBS. For confocal microscopy analysis, bacterial membrane was labeled with 2 μ M red fluorescent dye PKH26 (Sigma) and then washed twice in PBS. 10^5 HeLa-GFP-LC3 cells were cultured in the presence of 3×10^5 labeled GAS (MOI = 3) for 2 hr and then washed twice in PBS and analyzed by confocal microscopy (100 infected cells per experiment). For intracellular GAS viability assay, 10^5 HeLa cells were cultured in the presence of 50×10^5 GAS (MOI = 50) for 1 hr. Cells were then washed twice in complete RPMI medium containing 0.5% tannic acid (Sigma) and 20 μ g/ml ampicillin (Euromedex, Mundolsheim, France) for 15 min at 37°C. Cells were then incubated in 0.5% tannic acid and 20 μ g/ml ampicillin complete RPMI for 0, 1, or 3 hr. At the indicated time points, cells were washed in PBS and lysed in 200 μ l sterile distilled water, and serial dilutions of cell lysate were plated on *Streptococcus*-selective medium plates (Oxoid, Dardilly, France). The number of intracellular alive GAS was determined by counting colony-forming units on plates.

SUPPLEMENTAL DATA

Supplemental Data include ten figures and can be found with this article online at [http://www.cell.com/cell-host-microbe/supplemental/S1931-3128\(09\)00315-1](http://www.cell.com/cell-host-microbe/supplemental/S1931-3128(09)00315-1).

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