Rapid Degradation of the Complement Regulator, CD59, by a Novel Inhibitor*

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Background: Surface expression of complement inhibitors, such as the glycosylphosphatidylinositol-anchored protein CD59, prevent complement-dependent lysis of cancer cells.

Results: The non-toxic domain-4 of the bacterial toxin intermedilysin (rILYd4) blocks CD59 complement inhibitory activity.

Conclusion: rILYd4 induces CD59 internalization and rapid degradation in lysosomes in non-small lung carcinoma cells.

Significance: CD59 serves as a model for glycosylphosphatidylinositol-anchored protein trafficking and rILYd4 shows potential for immunotherapy.

There is increased interest in immune-based monoclonal antibody therapies for different malignancies because of their potential specificity and limited toxicity. The activity of some therapeutic monoclonal antibodies is partially dependent on complement-dependent cytolysis (CDC), in which the immune system surveys for invading pathogens, infected cells, and malignant cells and facilitates their destruction. CD59 is a ubiquitously expressed cell-surface glycosylphosphatidylinositol-anchored protein that protects cells from CDC. However, in certain tumors, CD59 expression is enhanced, posing a significant obstacle for treatment, by hindering effective monoclonal antibody-induced CDC. In this study, we used non-small lung carcinoma cells to characterize the mechanism of a novel CD59 inhibitor: the 114-amino acid recombinant form of the 4th domain of intermedilysin (rILYd4), a pore forming toxin secreted by Streptococcus intermedius.

We compared the rates of internalization of CD59 in the presence of rILYd4 or anti-CD59 antibodies and determined that rILYd4 induces more rapid CD59 uptake at early time points. Most significantly, upon binding to rILYd4, CD59 is internalized and undergoes massive degradation in lysosomes within minutes. The remaining rILYd4-CD59 complexes recycle to the PM and are shed from the cell. In comparison, upon internalization of CD59 via anti-CD59 antibody binding, the antibody-CD59 complex is recycled via early and recycling endosomes, mostly avoiding degradation. Our study supports a novel role for rILYd4 in promoting internalization and rapid degradation of the complement inhibitor CD59, and highlights the potential for improving CDC-based immunotherapy.

The complement system is a complex, multiprotein cascade in the circulation that continuously surveys for potential invading pathogens and infected cells. This essential arm of the innate immune system targets the membranes of invading pathogens for lysis. An antibody-dependent classical pathway and an antibody-independent alternative pathway ultimately lead to the deposition of complement proteins, known collectively as the membrane attack complex, on the target membrane (reviewed in Ref. 1). This allows the generation of pores that cause the destruction of pathogens or pathogen-infected cells. Mammalian cells are protected against the pore-forming complement by an array of membrane-bound complement-regulatory proteins, including CD35, CD46, and the glycosylphosphatidylinositol-anchored proteins (GPI-AP) CD55 and CD59 (2). Among these regulators, CD59 is the most important inhibitor for restricting membrane attack complex formation by binding to C8 and C9 and preventing C9 polymerization and insertion into membranes (3, 4).

CD59, a GPI-AP of ~20 kDa, is ubiquitously expressed in all human tissues and circulating cells (3). Rare cases of CD59 deficiency result in paroxysmal nocturnal hemoglobinuria (5, 6), a complement-induced anemia caused by the destruction of red blood cells in the bloodstream in the absence of CD59 protection, highlighting the essential function of this protein.

Immune surveillance by the complement system is widely believed to play a role in eliminating tumor cells (2). For this reason, there is growing interest in the use of immune-based therapies to target tumor cells. However, due to its role in pre-

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6 The abbreviations used are: GPI-AP, glycosylphosphatidylinositol-anchored protein; CDC, complement-dependent cytolysis; NSCLC, non-small cell lung carcinoma; PM, plasma membrane; GEEC, GPI-AP-enriched early endosomal compartments; MEM, minimal essential medium.
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venting complement-based lysis (reviewed in Ref. 2), CD59 expression poses a significant obstacle in the effective use of monoclonal antibody-induced complement-dependent cytoly-
sis (CDC) (7, 8). In parallel with the notion that CD59 expres-
sion might hamper CDC-based cancer immunotherapy, studies have demonstrated that expression levels of complement inhib-
itors are frequently higher in tumors than in cells from the surrounding tissue (9–13). This supports the idea that CD59 expression interferes with the killing of tumor cells by prevent-
ing immune complement-based lysis (reviewed in Refs. 14–17). For example, down-regulation of CD59 and complement system factor H allows sensitization of non-small cell lung carci-
noma (NSCLC) cells and their elimination via the complement system (18). Further support for this idea comes from studies demonstrating that expression of CD59 and CD55 on NSCLC cells was highly correlated with the histology and prognosis of the disease (9, 11).

The potential involvement of CD59 in tumor progression has led to the development of a new strategy for treatment of NSCLC. Recent studies have shown that antibody-based neu-
tralization of CD59 increases the efficacy of anti-Her2/neu antibody (herceptin/trastuzumab) treatment of NSCLC (19, 20), allowing more effective activation of CDC against these tumor cells (8, 21, 22). Moreover, neutralizing antibodies against CD55 and CD59 targeted to lymphoma cells in vitro enhance the therapeutic effect of Rituximab (22), but the lack of highly potent inhibitors for hCD59 limits its therapeutic appli-
cations. Consequently, alternative and effective new methods for CD59 neutralization have been a priority for researchers.

A new strategy for the attenuation of CD59 surface levels takes advantage of a protein called intermedilysin, a naturally occurring bacterial toxin. Intermedilysin is a pore-forming toxin, secreted by Streptococcus intermedius, and it binds with high affinity exclusively to the complement-binding site of human CD59 (23, 24). Recently, a novel CD59 inhibitor has been developed, which consists of a 114-amino acid recombi-
nant form of the 4th domain (d4) of intermedilysin (rILYd4).

rILYd4 is responsible for all of the binding to CD59, but it does not induce lysis (25). Thus, by binding to CD59, rILYd4 pre-
vents CD59 from interfering with the complement pore form-
ing membrane attack complex, thereby sensitizing the cells to CDC. The new studies with rILYd4 were designed to target CD59 at the surface of a variety of tumor cells in combination with monoclonal antibody treatment and demonstrated enhanced susceptibility of the tumor cells to CDC (8, 26, 27).

Despite the clinical potential of rILYd4, little is known of its mechanism of action beyond its association with CD59 at the plasma membrane (PM). Although it has been speculated that some of its inhibitory activity may be attributed to its steric interference with the complement proteins, its impact on both the localization of CD59 to the PM and on its subcellular itin-
erary have not been addressed. Herein, we provide evidence that rILYd4 moderately accelerates internalization of CD59 through a pinocytic pathway. Importantly, we demonstrate that a significant portion of CD59 that is associated with rILYd4 (rILYd4-CD59) undergoes rapid degradation in lysosomes, whereas the remaining internalized rILYd4-CD59 complexes recycle to the PM and are shed from the cell. In comparison, when internalization of CD59 was induced through anti-CD59 antibody binding, the antibody-CD59 complex entered an endocytic pathway that traversed the early and recycling endos-
omes, mostly avoiding degradation. Our study supports a novel role for rILYd4 in promoting rapid internalization and degradation of the complement inhibitor CD59, and highlights the potential of this inhibitor.

EXPERIMENTAL PROCEDURES

Cell Lines—H1650 NSCLC and HeLa cells were purchased from ATCC. NSCLC were grown in RPMI 1640 complete media containing 10% FBS, 2 mM glutamine, 1 x sodium pyru-
vate, 20 mM HEPES, 1 x MEM non-essential amino acids, 100 units/ml of penicillin, 100 units/ml of streptomycin, and 55 μM 2-mercaptoethanol.

Antibodies and Reagents—His-rILYd4 was produced and described previously in Ref. 25. All experiments in this study were carried out with 25 μg/ml of His-rILYd4, unless otherwise noted. Mouse monoclonal MEM-43 ascites antibody against CD59 was a generous gift of Dr. V. Horejsi (Academy of Sciences of the Czech Republic, Prague, Czech Republic; also used in Refs. 28 and 29). This antibody recognizes rILYd4-CD59 complex with similar affinity as recognizing CD59 alone (see the antibody comparison study of MEM-43 and 2 other monocl-

onal anti-CD59 antibodies for recognition of the complex in Fig. 1). Hence, detection of the complex by immunofluorescence, flow cytometry, and dot blot were all carried out with MEM-43 antibody. Commercial H19 anti-CD59 antibody (BD Biosciences) and H-85 (Santa Cruz) were used in this study for immunoblotting. Other commercial antibodies used were: mouse anti-His (Abcam), mouse anti-actin (Novus Biologicals, Inc.), rabbit anti-Cdc42 (Santa Cruz Biotechnology), goat anti-mouse horseradish peroxidase (HRP) (Jackson Immunoresearch Laboratory, Inc.), donkey anti-rabbit HRP (GE Healthcare), Alexa 568 goat anti-mouse, Alexa 405 goat anti-rabbit and Alexa 647 goat anti-mouse F(ab)₂ (Invitrogen), rabbit anti-Rab11 (U. S. Biologicals), rabbit anti-EEA1 (Cell Signaling), rabbit anti-lamp1 (Novus), and rabbit anti-caveolin (Cell Signaling). EZ-link NHS-LC-Biotin was purchased from Pierce. Leupeptin and cycloheximide were purchased from Fisher and Sigma, respec-
tively. Alexa 555 and 488 labeling kits and 4',6-diamidino-2-
phenylindole (DAPI) were from Invitrogen.

Immunoblotting—NSCLC cells were harvested and lysed on ice for 30 min in lysis buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, and protease inhibitor mixture (Roche Applied Science). Total protein level in the lysate was quan-
tified by Bio-Rad protein assay (Bio-Rad) for equal protein loading on gels. Protein samples were separated on 13% SDS-
PAGE, followed by immunoblotting with appropriate antibodies.

Immunofluorescence and Uptake Assays—NSCLC cells grown on coverslips were incubated with 25 μg/ml of His-rILYd4 in complete media for the indicated time at 37 °C. Cells were then fixed with 4% (v/v) paraformaldehyde in PBS for 10 min. Fixed cells were incubated with primary antibodies pre-
pared in staining solution containing 0.2% saponin (w/v) and 0.5% BSA (w/v) in PBS for 1 h at room temperature. After washes in PBS, cells were incubated with the appropriate fluo-
orochrome-conjugated secondary antibody for 30 min in stain-
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Cell grown on 100-mm dishes were suspended and allowed to internalize His-rILYd4 for 10, 20, or 30 min at 37 °C, or kept in media without His-rILYd4 under similar conditions. At the end of each time point, cells were cooled on ice to stop internalization, washed with cold PBS, and biotinylated on ice (0.3 mg/ml). After lysis and immunoprecipitation with anti-CD59 (MEM-43), eluates were separated on SDS-PAGE, and biotinylated CD59 was detected with streptavidin-HRP. Actin from the lysate was used as a loading control. For CD59 uptake, cells were incubated with lysate was used as a loading control. For co-immunoprecipitation, CD59 was detected with streptavidin-HRP. Actin from the lysate was used as a loading control. For co-immunoprecipitation of His-rILYd4 and endogenous CD59, cells were lysed in buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, and 0.5% Triton X-100. Cell lysates were pre-cleared with protein G beads at 4 °C for 1 h and then incubated with either mouse anti-His antibody or mouse IgG (control) overnight. Protein G beads were added to the lysate/antibody mixture for 4 h. After three washes with 0.1% Triton X-100, proteins were eluted from the protein G beads by boiling in the presence of 4× SDS sample buffer. Eluted proteins were then subjected to 13% SDS-PAGE.

Densitometry and Image Analysis—ImageJ software (rsbweb.nih.gov/ij/links.html) was used to measure pixel intensity and/or particles/cell. For analysis of vesicle size and number of vesicles (particles), images from 3 independent immunofluorescence experiments were used, taken at identical magnification, and containing 11–17 cells per image (4–5 such images per experiment). For particles/cell analysis, a threshold that defines a particle was set as 3–300 pixels. The total number of particles per field was divided by the number of cell nuclei (DAPI-stained). In the images obtained with anti-HA antibody (complexed with rILYd4, rendering this antibody incompatible with native, non-denatured rILYd4) and its CD59) and its endosomally bound CD59 complex was compared with the recognition of CD59 alone, using Alexa 647 goat anti-mouse F(ab)_2 (without detergent). At least 10,000 cells per sample were collected for flow cytometry analysis. Values from 3 independent experiments were normalized; BRIC without rILYd4 was set as 100% antibody binding.

Transfection and siRNA Treatment of NSCLC Cells—Because transfection of NSCLC cells with FuGENE HD (Roche Applied Science) interfered with rILYd4 uptake, the following protocol was used. After a 6-h transfection, cells were washed to remove transfection reagent, and incubated at 37 °C in complete media for an additional 10 h to recover from transfection and were now competent to uptake rILYd4.

siRNA treatment was carried out with Oligofectamine (Invitrogen) for 72 h according to the manufacturer’s protocol using 3 μM oligonucleotide (Dharmacon, Lafayette, CO). Four specific oligonucleotides (On-Target SMART pool) were directed at human Cdc42 (CGGAAUAUGUAACCUGUGU, GCAGUCACAGUAUGAUUG, GAUGACCCCUCAUCUUG, and CUGCAGGCGAACAGGUAUA). Four specific oligonucleotides (On-Target SMART pool) were directed at human CD59 (GAAGGACCUGUAACCUU, CUAAACUGUCUAAACCA, GUAACUUAAACGAACAGCU, and UACCA-AAGCUGGGGUUACAA).

RESULTS

Co-localization of Internalized rILYd4 with CD59-containing Endosomes—Our initial goal was to address whether rILYd4 impacts CD59 exclusively by inducing steric interference with the complement system or whether rILYd4 affects CD59 via additional mechanisms that might include internalization and/or degradation. To investigate the internalization of a complex containing CD59 bound to rILYd4 (rILYd4-CD59) and its subsequent endocytic pathways, we first established a detection system that would allow us to visualize and follow internalizing rILYd4-CD59 over time in NSCLC cells.

First, it was crucial to identify an anti-CD59 antibody that would recognize CD59 equally well when bound to rILYd4. To this aim, we tested the binding of 3 separate antibodies to native, non-denatured rILYd4-CD59 by flow cytometry (Fig. 1A). NSCLC cells were kept on ice in the absence or presence of His-tagged rILYd4 for 45 min to allow binding to surface-CD59 without internalization. After washing, cells were then either incubated on ice with anti-His antibodies (as a positive control to demonstrate rILYd4 binding; Fig. 1A, anti-His), or incubated with the following monoclonal anti-CD59 antibodies: H19, MEM-43, or BRIC. As shown, despite being a useful antibody for denatured CD59 in immunoblot analysis, the H19 antibody poorly recognized native CD59 whether or not the CD59 was complexed with rILYd4, rendering this antibody incompatible for immunofluorescence and flow cytometry experiments (Fig.
A. The BRIC antibody bound robustly to free CD59, but displayed significantly decreased binding to rILYd4/CD59 complexes as previously reported (25), also rendering this antibody incompatible for our studies. On the other hand, the MEM-43 antibody displayed a strong and identical binding to CD59, whether or not it was pre-bound to rILYd4. The identification of MEM-43 as an antibody that bound rILYd4/CD59 equally well to CD59 alone has provided us with a unique tool to conduct our studies with the native, non-denatured proteins.

rILYd4 internalization was primarily dependent on CD59, as siRNA depletion of CD59 (Fig. 1B) induced a dramatic decrease of intracellular His-rILYd4 at 3 and 10 min internalization (Fig. 1, see insets, and compare D to F and E to G; quantified as internalized particles per cell in C, which also includes a 15-min internalization time point). The very minimal uptake still observed (within 3 min) in the absence of CD59 might result from the ability of rILYd4 to interact with cholesterol (23), and potentially be taken up by spontaneous pinocytic mechanisms.

Prior to commencing our immunofluorescence-based studies on rILYd4/CD59 internalization and intracellular trafficking, our next priority was to distinguish between surface-residing rILYd4/CD59 and internalized complex. To this aim, we

FIGURE 1. rILYd4 is internalized in a CD59-dependent manner. A, the ability of 3 different monoclonal anti-CD59 antibodies to recognize native, non-denatured rILYd4/CD59 complex was tested by flow cytometry. NSCLC cells were kept on ice in the absence (white bars) or presence of His-tagged rILYd4 (gray bars) for 45 min to allow binding to surface-CD59 without internalization. After washing, cells were either incubated on ice with anti-His antibodies (to demonstrate rILYd4 binding; “anti-His” positive control on the left of the graph), or with the following anti-CD59 antibodies: H19, MEM-43, or BRIC. After fixation, all samples were stained with Alexa 647 goat anti-mouse F(ab)2 secondary antibody. The graph represents 3 independent experiments, which were normalized (BRIC with no rILYd4 was set as 100% binding to CD59). Error bars denote S.D. B–G, CD59 depletion delays His-rILYd4 internalization. Cells grown in 35-mm dishes were treated with scrambled- or CD59-siRNA for 3 days. For B, cells were lysed and the level of CD59 was detected by immunoblot. Actin was used as a loading control. For D–G, scrambled- or CD59-siRNA treated cells were incubated with His-rILYd4 for 3 (D and F) or 10 min (E and G). After fixation, cells were stained with Alexa 488-conjugated anti-His antibody. C, 3 independent experiments similar to D–G are quantified, including a 15-min uptake time point. Internalization of rILYd4/CD59 complex is expressed as particles per cell. Bars, 10 μm.
FIGURE 2. Distinguishing between surface and internalized rILYd4-CD59 complex. To distinguish between surface-residing rILYd4-CD59 and internalized complex by immunofluorescence, the following protocol was designed: A–F, the panels visualize surface-CD59 and rILYd4: cells were incubated simultaneously with His-ILYd4 and anti-CD59 antibody (MEM-43) for 10 min at 37 °C. As demonstrated in Fig. 1, MEM-43 does not compete with rILYd4, and thus was used to label the surface-CD59 pool. After 10 min co-uptake, the cells were stripped and fixed (D–F) or fixed without stripping (A–C) prior to immunostaining with Alexa 568 goat anti-mouse in the absence of saponin. Note the rapid disappearance of rILYd4 from the cell-surface in B. G–L, the panels visualize intracellular CD59 and rILYd4. In an identical 10-min co-uptake of His-rILYd4 and Alexa 555-conjugated anti-CD59 (MEM-43) followed by stripping, internal CD59 and His-rILYd4 (strip-resistant and in the presence of saponin) were detected on tiny punctae. J–L, a single cell is shown at higher magnification. Note that His-rILYd4 and internalized CD59 are highly co-localized in L (white arrows).
at 37 °C rendered the stripping step unnecessary, and hence, immunofluorescence experiments in this study did not require this additional step.

Having calibrated a controlled system to follow internalization of rILYd4-CD59, we next asked whether rILYd4-CD59 complexes are internalized to common endocytic structures. To this aim, we incubated NSCLC cells with His-rILYd4 for 15 min at 37 °C, followed by fixation. Cells were then stained with Alexa 488-conjugated anti-His (which partially stains the nucleus) and Alexa 555-conjugated anti-CD59 antibodies. Right images are insets. Pink arrows indicate the co-localization of His-rILYd4 with CD59 (note that the CD59 stain in D is substantially reduced compared with B). E, the degree of co-localization between His-rILYd4 (internalized for 15 min) and endogenous CD59 was quantified by analyzing rILYd4-positive vesicles of 20 cells from 3 different experiments. Standard error is shown. F, cells grown on 60-mm dishes were treated either without or with His-rILYd4 for 15 min at 37 °C. Lysate was separated by SDS-PAGE and immunoblotted with anti-CD59, anti-His, and anti-actin. G, after lysis, anti-His was used to pull-down endogenous CD59. Isotype-compatible IgG was used as a control for anti-His. Immunoblotting was done with anti-CD59 and anti-His. Bars, 10 μm.

FIGURE 3. Internalized rILYd4 co-localizes and remains bound to CD59. A–D, H1650 cells grown on coverslips were incubated either without (A and B) or with His-rILYd4 (C and D) for 15 min at 37 °C, followed by fixation. Cells were then stained with Alexa 488-conjugated anti-His (which partially stains the nucleus) and Alexa 555-conjugated anti-CD59 antibodies. Right images are insets. Pink arrows indicate the co-localization of His-rILYd4 with CD59 (note that the CD59 stain in D is substantially reduced compared with B). E, the degree of co-localization between His-rILYd4 (internalized for 15 min) and endogenous CD59 was quantified by analyzing rILYd4-positive vesicles of 20 cells from 3 different experiments. Standard error is shown. F, cells grown on 60-mm dishes were treated either without or with His-rILYd4 for 15 min at 37 °C. Lysate was separated by SDS-PAGE and immunoblotted with anti-CD59, anti-His, and anti-actin. G, after lysis, anti-His was used to pull-down endogenous CD59. Isotype-compatible IgG was used as a control for anti-His. Immunoblotting was done with anti-CD59 and anti-His. Bars, 10 μm.

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Having calibrated a controlled system to follow internalization of rILYd4-CD59, we next asked whether rILYd4-CD59 complexes are internalized to common endocytic structures. To this aim, we incubated NSCLC cells with His-rILYd4 for 15 min at 37 °C (Fig. 3, C and D) and fixed them, and then used anti-His and anti-CD59 antibodies for detection of control and His-rILYd4-treated cells (Fig. 3, A–D). As expected, in the absence of His-rILYd4 (Fig. 3A), no His-rILYd4 was detected; only a weak background nuclear stain was observed. Also as expected under these conditions, CD59 was observed primarily at the PM (Fig. 3B). Incubation with His-rILYd4 led to the visualization of multiple internal punctae that coincided with CD59 (Fig. 3, C and D, see insets, arrows) indicate vesicles containing both CD59 and His-rILYd4). This co-localization persisted over a longer period of time (up to 80 min). Note that the endogenous CD59, as portrayed in Fig. 3, B and D, and the insets, resides essentially in 2 pools: (i) CD59, which initially localized to the PM, formed a complex with His-rILYd4, and was internalized, and (ii) the intracellular CD59 pool, which had no access to His-rILYd4. Hence, whereas a portion of CD59

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co-localized with His-rILYd4, a non-bound “free” CD59 was also detected (red channel in the inset merge), which represents the intracellular pool that did not bind rILYd4 on the PM. Quantification of internalized His-rILYd4-positive vesicles revealed that 93% of the peripheral endosomes containing internalized His-rILYd4 also contained CD59 (Fig. 3E). It is of particular interest that the overall intensity of CD59 was dramatically reduced upon rILYd4 treatment (compare Fig. 3, D to B, and lanes 1 and 2 in F, upper panel). This phenomenon is discussed in detail in Figs. 4–7.

The ability of rILYd4 to associate with surface-CD59 was previously established (8, 23). By co-immunoprecipitation we now demonstrate that this interaction is maintained post-endocytosis, following a 15-min incubation of rILYd4 at 37 °C. In Fig. 3G, immunoprecipitation of His-rILYd4 (lane 2, lower panel), pulled down CD59 (lane 2, upper panel), but not in the absence of rILYd4 (lane 1). As a control, isotype-compatible IgG pulled down neither protein (lanes 3 and 4). These data indicate that CD59 is internalized while bound to rILYd4.

**The Levels of Internalized rILYd4 Decrease Over Time**—To follow the intracellular itinerary of internalized His-rILYd4, we incubated cells with His-rILYd4 for 15 min, performed a “chase” in complete media for 80 min, and analyzed the intracellular distribution of His-rILYd4. As demonstrated after internalization in Fig. 4A (see inset), His-rILYd4 was observed in tiny peripheral vesicles. However, after an 80-min chase (Fig. 4B, see inset), there was a significant decrease in the intracellular His-rILYd4 signal, with the remaining His-rILYd4 still observed in small peripheral structures. The levels of internalized His-rILYd4 were further analyzed by immunoblotting. As shown in Fig. 4C, after an 80-min chase, His-rILYd4 decreased by over 95% compared with its initial level after a 15-min internalization. The loss of internalized His-rILYd4 over time has important ramifications for the potential manipulation of cellular CD59.

**rILYd4 Induces Decreased Surface and Total CD59 Levels**—Because the presence of CD59 on the cell surface protects cancer cells from complement-based cell lysis, we next inquired whether His-rILYd4 alters the presence of CD59 at the cell surface. The first question was whether His-rILYd4 has any impact on surface-CD59 display, and whether it affects CD59 endocytosis. Accordingly, we measured the disappearance of surface-CD59 from the PM upon a short incubation with rILYd4. NSCLC cells were incubated at 37 °C with or without 25 μg/ml of rILYd4 for the indicated times (Fig. 5A). At each time point, cells were cooled on ice to stop internalization, and then briefly surface biotinylated on ice and lysed. Anti-CD59 (MEM-43) was then used to immunoprecipitate CD59, which was subjected to SDS-PAGE. Streptavidin-horseradish peroxidase detected the surface-CD59. Essentially, “no rILYd4”-treated cells represent a continuous incubation at 37 °C, and all three time points serve as untreated controls for zero time (because 10 min with no treatment does not differ from 20 or 30 min with no treatment).

As shown, whereas surface-CD59 remained largely unchanged in the control samples (Fig. 5A, left panel), a dramatic reduction in surface-CD59 was noticeable as early as 10 min following incubation with rILYd4 (Fig. 5A; compare 10 min + rILYd4 to 10 min “no rILYd4”). The reduction in biotinylated CD59 was even more robust after 20 and 30 min of incubation with rILYd4 (Fig. 5A, right panel). In parallel, the total CD59 level was also measured with lysates from these same samples (blotted with anti-CD59) and mirrored a similar reduction in total CD59.7 Quantification of 3 independent experiments (in Fig. 5B) suggests up to a 30-fold drop in surface-CD59 at 30 min.

In agreement with the biotinylation data, immunofluorescence staining of surface-CD59 (with MEM-43) revealed a dramatic reduction in surface-CD59 levels upon 15 min incubation with rILYd4 (compare 0, 12.5, 25 μg/ml of rILYd4; Fig. 5B, left panel). Collectively, these data imply that rILYd4 induces a rapid reduction in cell surface levels of CD59.

To characterize the concentration of rILYd4 required to induce loss of CD59, we used a range of concentrations from 0 to 25 μg/ml. As depicted in Fig. 6A (quantified in B), there is a dose-dependent effect whereby 25 μg/ml of His-rILYd4 had a significantly more potent effect than the next highest concentration (12.5 μg/ml). We further characterized the effect of rILYd4 on CD59 loss by comparing the His-tagged version with untagged rILYd4. As demonstrated (Fig. 6C), although the
FIGURE 5. His-rILYd4 induces CD59 disappearance from the cell surface.

A, the surface level of CD59 was measured by immunoprecipitating surface-biotinylated CD59. Cells grown on a 100-mm dish were suspended and allowed to internalize His-rILYd4 for the indicated time at 37 °C (right panel), or kept in media without His-rILYd4 under similar conditions (left panel). At the end of each time point, cells were cooled down on ice to stop internalization, washed, and then biotinylated on ice. Following lysis and immunoprecipitation with anti-CD59 (MEM-43), samples were separated on SDS-PAGE, and biotinylated CD59 was detected with streptavidin-HRP. Actin from the lysate was a loading control. B, densitometry analysis of 3 independent biotinylation experiments. Standard deviation is shown. C and D, immunofluorescence detection of surface-CD59. Cells grown on coverslips were incubated either without (C) or with (D) His-rILYd4 for 15 min at 37 °C, followed by fixation. Surface CD59 was detected with Alexa 555-conjugated anti-CD59 antibodies in the absence of saponin. Bar, 10 μm.

untagged rILYd4 has a stronger inhibitory effect on complement-derived cytotoxicity (31), both His-rILYd4 and rILYd4 massively induced CD59 loss. To determine whether the effect of rILYd4 on CD59 occurs in other cell types, we carried out experiments with HeLa cells. Although CD59 levels are not as robust as in NSCLC cells, treatment with rILYd4 similarly induced rapid loss of CD59 in these cells (Fig. 6D). Similar results were obtained with the human prostate cancer cell line, PC3.7.

To examine whether degradation is a possible explanation for the disappearance of CD59 from the cell when bound to rILYd4, cells were pre-treated with the lysosomal inhibitor, leupeptin, and then incubated with His-rILYd4 for 15 min at 37 °C. As we have demonstrated, rILYd4 treatment induced massive CD59 loss (Fig. 6, E, compare lanes 1 and 2, upper panel, quantified in F). However, upon leupeptin treatment, CD59 was typically restored to 70–80% of its original expression level (Fig. 6, E, lane 3, quantified in F). In contrast, pre-treatment with the proteasomal inhibitor MG132 did not restore CD59 protein levels, suggesting that upon its association with rILYd4, CD59 undergoes rapid proteolysis that is not proteasomal degradation.

Due to the extent of degradation after just 15 min, we consistently observed only a small portion of the complexes in lysosomes (Fig. 7C, inset, arrow marks rILYd4-CD59-containing vesicles also positive for LAMP1, a lysosomal marker (in white)). Upon leupeptin treatment, a 3-fold increase in the number of rILYd4-CD59-containing vesicles could be traced in LAMP1-lysosomes (Fig. 7D, inset, quantified in Fig. 7B). The remaining (non-degraded) rILYd4-CD59 were localized to peripheral carriers (Fig. 7, C and D, yellow vesicles). These data support the idea that the loss of CD59, triggered by rILYd4, may result mainly from rapid lysosomal degradation. Such unusually rapid lysosomal degradation caused by a GPI-AP-ligand has not, to the best of our knowledge, been previously reported.

We next examined whether rILYd4-induced lysosomal degradation of CD59 persists even upon rILYd4 removal from the media (“washout”). To address this, after a 15-min incubation in the presence or absence of His-rILYd4, cells were “chased” in complete media for 80 min (Fig. 6E, lanes 4–6, quantified in F). Although the level of CD59 remained low after an 80-min washout (Fig. 6E, lane 5 compared with lane 2, at “pulse”), leupeptin similarly restored CD59 levels as it did during the 15-min “pulse” (Fig. 6E, lane 6). Quantification of 4 independent experiments (in Fig. 6F) consistently revealed a 3–6-fold drop in CD59 upon rILYd4 binding, and a 70–80% restoration when lysosomal activity was inhibited.

As GPI-AP can undergo cleavage and be shed from the PM (32), we tested whether CD59 is shed into the supernatant as a result of its association with rILYd4. In the course of a 15-min incubation with rILYd4, during which the massive decrease in CD59 occurred, only residual shedding of CD59 was detected in the supernatant (Fig. 7A, lanes 3 and 4). Importantly, His-rILYd4 did not increase CD59 shedding, further attributing CD59 loss to lysosomal degradation. In addition, we tested the possibility that leupeptin might also impact shedding. Pre-treatment with leupeptin, followed by incubation with His-rILYd4 for 15 min indeed resulted in modestly decreased shedding of CD59 (Fig. 7A, lane 5; see “Discussion”). Moreover, even after washing out the rILYd4 and incubating the cells for an additional 80 min, rILYd4 had no impact on CD59 shedding (Fig. 7E, left panel), although some of the complex did recycle and undergo shedding during the 80-min chase (Fig. 7E, right panel). Newly synthesized CD59 did not affect the above result, as cycloheximide did not alter CD59 shedding in the presence or absence of rILYd4 (Fig. 7F). These data demonstrate that although constitutive shedding of CD59 occurs, binding to rILYd4 does not alter this shedding. Therefore, the major mechanism for loss of CD59 upon rILYd4 binding occurs via lysosomal degradation.

rILYd4-CD59 Complexes Enter the Cell Rapidly and Partially Co-localize with Fluid-phase Cargo—GPI-APs enter cells via clathrin-independent pathways (often termed clathrin independent carriers (CLIC) (33) or through Arf6- or caveolin1-independent mechanisms (reviewed in Ref. 34). Accordingly, we sought to define the requirements for the endocytosis of rILYd4-CD59 complexes.

Accumulation of GPI-AP molecules in caveolae is thought to result from a degree of clustering by the ligand at the cell surface (35). According to our observations, however, it is
unlikely that rILYd4 binding drives CD59 into caveolae, as Caveolin1 did not coincide with internalized rILYd4 at either 3 or 10 min post-internalization. To further characterize the mode of rILYd4/CD59 complex internalization, we compared it to a well established system for inducing CD59 uptake: the use of anti-CD59 antibody (36). The binding between CD59 and the antibody (MEM-43 in this study) is maintained throughout the endocytic pathway, and is thus referred as a “complex” (36).

Quantitative immunofluorescence was used to measure the rate of CD59 internalization induced either by His-rILYd4 or anti-CD59 following 1–10-min incubation. Vesicles containing the internalized complexes were scored as “particles.” As demonstrated in Fig. 8A, the number of internalized particles per cell was greater at early time points (1–5 min) with His-rILYd4 incubation. However, at 10 min of uptake this difference was no longer apparent (Fig. 8A). As previously shown in Fig. 3F for His-rILYd4-treated cells, starting at ~15 min, CD59 begins to

![FIGURE 6. rILYd4 induces CD59 degradation in lysosomes. A, cells grown on 35-mm dishes were incubated with rILYd4 at the indicated concentrations for 15 min. Cells were then lysed and subjected to 13% SDS-PAGE, followed by immunoblotting with antibodies against CD59 and actin (as a loading control). B, 3 individual experiments from A were quantified. C, HeLa cells were either untreated, or treated with 25 μg/ml of His-rILYd4 or 25 μg/ml of untagged rILYd4 for 15 min at 37 °C. Cells were then lysed and CD59 levels were detected by immunoblot. D, HeLa cells were incubated either without or with untagged rILYd4 for 15 min at 37 °C. Cells were then lysed and the CD59 level was analyzed by immunoblot. E, cells were either pretreated with 500 μg/ml of leupeptin for 3 h at 37 °C (lanes 3 and 6) or left untreated (lanes 1, 2, 4, and 5). His-rILYd4 was added for 15 min (lanes 2, 3, 5, and 6). Control samples did not include incubation with His-rILYd4 (lanes 1 and 4). Cells were lysed and the levels of CD59, His-rILYd4, and actin (loading control) were detected by immunoblotting and quantified (3 independent experiments) in F.]

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undergo degradation. However, in stark contrast, this degradation does not occur if CD59 is internalized via antibody binding (Fig. 8B). Thus, the rapid degradation of CD59 (only upon rILYd4-induced internalization) might explain how the number of internalized particles per cell observed after 10 min in rILYd4-treated cells is similar to that in anti-CD59-treated cells (Fig. 8A).

Although we were unable to determine whether anti-CD59 and rILYd4 induce CD59-internalization by different modes, the size of CD59-containing vesicles for each mode of uptake differed. Whereas internalized CD59-containing particles increase in size over time when internalized upon antibody incubation, they remain small and constant in size with His-rILYd4 treatment (Fig. 8C). This difference in particle size is evident in the insets of Fig. 8, D and E, where rILYd4-containing endosomes remain small after 15 min uptake (Fig. 8D, inset in green), whereas anti-CD59–CD59 complexes reside in much larger structures (Fig. 8E, inset in green).

**FIGURE 7.** Shedding of CD59 does not contribute to rILYd4-induced CD59 cell-surface loss. A, cells grown on 35-mm dishes were either pre-treated with leupeptin for 3 h at 37 °C (lane 5) or left untreated (lanes 1–4). His-rILYd4 was then added for 15 min (lanes 2, 4, and 5), cell supernatants were collected and loaded on dot-blotter, and immunoblotting was done with mouse anti-CD59 antibody (MEM-43) followed by goat anti-mouse HRP. B–D, rILYd4–CD59 complexes reach lysosomes. H1650 cells were either untreated (C and inset) or pre-treated with leupeptin for 3 h (D and inset). Cells were then incubated with His-rILYd4 for 15 min at 37 °C (a time point at which degradation already occurs; see Fig. 3F). His-rILYd4 and CD59 were detected with directly conjugated antibodies: Alexa 488-conjugated anti-His and Alexa 555-conjugated anti-CD59. Lysosomes were stained with rabbit anti-LAMP1 antibody, followed by Alexa 405 goat anti-rabbit secondary antibody. White arrows in insets denote triple-stained vesicles. B, triple-stained vesicles containing His-rILYd4, CD59, and LAMP1 in untreated versus leupeptin-treated cells, were quantified from 3 independent experiments by Pearson’s coefficient. E, after incubation with or without His-rILYd4 for 15 min at 37 °C, cells were washed and chased in complete media for 0, 20, or 80 min as indicated. Supernatant was collected at each time point and dot blotted. Shed CD59 (left panel E), and shed His-rILYd4 (right panel E), were detected with anti-CD59 and anti-His antibodies respectively. F, the above experiment was performed (80 min chase) in the presence of either DMSO or 150 μg/ml of cycloheximide. Error bars denote S.E. Bars, 10 μm.
GPI-AP-enriched early endosomal compartments (GEECs) are specialized endosomes that contain a large portion of internalized GPI-AP, bacterial toxins, and fluid phase (33, 37). Although the canonical regulators of GPI-AP endocytosis into GEECs had little or no impact on the internalization of rILYd4/H18528 CD59, there are multiple modes of GPI-AP internalization into the CLICs/GEECs pathway (34). For example, GPI-APs reach uncoated endosomes that also uptake cholera toxin (33) and fluid phase (37). Accordingly we performed a sequential uptake of either rILYd4 or anti-CD59, followed by a short incubation with the fluid-phase marker, Alexa 555-BSA. After 15 min internalization, His-rILYd4 was present in BSA-positive vesicles at the cell periphery (Fig. 8D, see inset), displaying a Pearson’s coefficient of 0.58 (Fig. 8F, 58% co-localized). This co-localization persisted for about 20 min; at later time points, BSA was observed in lysosomes,7 whereas His-rILYd4 remained in these small peripheral structures for an additional ~80 min before its intensity diminished (see Fig. 4B). Likewise, antibody-induced CD59 internalization also co-localized with BSA (Fig. 8E and inset), albeit to a somewhat greater degree (80% co-localization; quantified in Fig. 8F). Taken together, although we cannot rule out differential routes of internalization, these observations lead us to suggest that post-internalization sorting and trafficking of rILYd4-CD59 complexes are likely the primary cause of

**FIGURE 8.** rILYd4-CD59 complexes internalized more rapidly than anti-CD59-CD59 complexes. A and C, cells were incubated for 1, 3, 5, and 10 min with either 25 μg/ml of His-rILYd4 or 25 μg/ml of anti-CD59, and the number of internalized CD59- and His-rILYd4-containing vesicles was measured. Their mean size (area) is summarized in C. B, cells were incubated in the absence (−) or presence of CD59 antibody (+) for 15 min, lysed, and immunoblotted for total CD59 levels. Note the lack of degradation here, compared with Fig. 3F. Actin served as a loading control. D and E, cells were incubated with either 25 μg/ml of His-rILYd4 (D) or 25 μg/ml of mouse anti-CD59 antibody (MEM-43) (E) for 5 min at 37 °C, washed three times with PBS, then incubated with 0.5 mg/ml of Alexa 555-BSA for 10 min, followed by fixation. Fixed cells were stained with Alexa 488-conjugated anti-His antibody and DAPI. Insets show co-localization between BSA and CD59 (note the difference in particle size between D and E; see insets). F, graph depicting Pearson’s coefficient for the degree of co-localization between CD59 and BSA upon either His-rILYd4 or anti-CD59 incubation. Bar, 10 μm.
Rapid CD59 Degradation Induced by a Novel Inhibitor

Rapid CD59 Degradation Induced by a Novel Inhibitor

The rapid CD59 degradation (rather than the mode of the internalization pathway).

rILYd4 and Anti-CD59 Antibodies Direct CD59 to Different Sorting Pathways—The small GTPase Cdc42 is a crucial trafficking regulator that oversees delivery of GPI-AP into GEECs (37, 38) and controls normal vesicle transport, sorting, and polarity (39, 40). Here we assessed the potential involvement of Cdc42 in post-internalization trafficking of rILYd4/H18528CD59 complexes versus anti-CD59/H18528CD59 complexes. NSCLC cells were treated with siRNA to knockdown the expression of endogenous Cdc42 (Fig. 9A). For anti-CD59-treated NSCLC cells, the loss of Cdc42 had little impact on the number of internalized particles observed after 15 min uptake (Fig. 9, D and E, see insets, quantified in B, left bars). At this time point, treatment with rILYd4 resulted in a decreased number of overall cellular particles remaining, likely due to the onset of rapid degradation (Fig. 9, F and G, quantified in B, hatched brown bar). Importantly, Cdc42 depletion caused only a small reduction in overall CD59-containing particles per cell compared with scrambled siRNA (Fig. 9B, compare orange with brown bars), again consistent with enhanced CD59 degradation. Indeed, Cdc42 depletion did not prevent the rapid lysosomal degradation of CD59 observed upon rILYd4 incubation (Fig. 9C, upper panel, compare lanes 3 and 4 with 1 and 2), suggesting that Cdc42 does not play a major role in the sorting of rILYd4/CD59 or CD59-antibody complexes after internalization.

There is growing number of translational-oriented studies using antibodies to neutralize CD59 and thus enhance the efficacy of complement-based immunotherapy (8, 21, 22). This prompted us to next investigate whether the anti-CD59 antibody MEM-43 directs CD59 to different intracellular destinations than those directed by rILYd4.

To conduct this comparison, we incubated NSCLC cells with MEM-43 anti-CD59 antibody for 15 min and used a brief acid strip to remove surface-remaining antibody. Cells were then fixed and co-stained with EEA1 to assess whether CD59 reached early endosomes. As we and others have previously shown (41, 42) antibody-bound internalized CD59 was predominantly found in early endosomes, as judged by its significant co-localization with endogenous EEA1 (Fig. 10A, pink structures in inset, quantified in C). On the other hand, following an identical incubation time with His-ILYd4, peripheral vesicles containing rILYd4/CD59 completely lacked EEA1 (Fig. 10B, see CD59 (in red) and EEA1 (in blue) and compare CD59/EEA1 insets in A and B, quantified in C). Importantly, HisrILYd4 still maintained its association with CD59 (Fig. 10B, inset rILYd4/CD59, in yellow). Even at earlier time points (3 and 10 min) rILYd4/CD59 was excluded from early endosomes (as stained with EEA1, data not shown). This implies that post-internalization, His-rILYd4 and anti-CD59 antibodies direct CD59 to separate destinations within the cell.

FIGURE 9. Internalized His-rILYd4 undergoes degradation in a Cdc42-independent pathway. A and C, cells were treated with either scrambled- or Cdc42-siRNA for 3 days. For A, after lysis the level of endogenous Cdc42 was assessed by immunoblot. For C, cells were incubated with His-ILYd4 for 15 min (lanes 3 and 4) or remained untreated (lanes 1 and 2), then lysed and subjected to SDS-PAGE, immunoblotted with anti-CD59, -His, and -actin (as loading control). D–G, cells treated as in A were incubated for 15 min with either 25 μg/ml of His-rILYd4 (D and E) or 25 μg/ml of mouse anti-CD59 antibody (MEM-43) (F and G; nuclear stain is nonspecific). Fixed cells were stained with anti-His (in F and G) and Alexa 488 goat anti-mouse secondary (D–G). B, 3 experiments similar to D–G were quantified. CD59 internalization is analyzed as the mean number of particles per cell. Bar, 10 μm.
To further compare the distinct intracellular pathways of CD59 traversed upon rILYd4- versus anti-CD59-induced internalization, we studied the recycling pathway under each mode of internalization. A key regulator of GPI-AP recycling is the small GTPase Rab11, a well-established marker of the recycling endosomes (43). We asked whether there are differences in CD59 localization to Rab11-containing recycling endosomes upon its internalization via rILYd4 or antibody. To this aim,
CD59 was internalized using anti-CD59 for 15 min, and cells were acid-stripped and then chased for 40 min to allow recycling. The co-localization of internalized CD59 with Rab11 was extensive (Fig. 11A, co-stain in pink, quantified in C). However, upon His-rILYd4 binding (under identical conditions to those in Fig. 11A), the remaining non-degraded complex did not co-localize with Rab11 (Fig. 11B, inset CD59/Rab11, compare pink punctae to the parallel inset in A, quantified in C). Throughout the experiment, His-rILYd4 still maintained its association with CD59 (Fig. 11B, inset rILYd4/CD59, yellow). These data imply that recycling of the rILYd4-CD59 complex might occur from endosomes lacking Rab11. Therefore, the possibility that...
the complex enters the “fast recycling” pathway was also studied: Rab4, a GTPase that was linked to the fast recycling pathway (44), did not co-stain with the rILYd4-CD59 complex,7 and its involvement in the recycling of rILYd4-CD59 remains unclear. Taken together, two distinct CD59 endocytic itineraries were observed, and the fate of CD59 depends upon its ligand. Moreover, differences in post-endocytosis events such as sorting and recycling might explain the unusual intracellular route of CD59 upon rILYd4 binding.

DISCUSSION

Despite modest advances in the treatment of NSCLC with anti-growth factor monoclonal antibodies to elicit CDC, immune complement regulatory proteins such as CD59 still present a significant obstacle to effective treatment. Thus far, attempts to neutralize CD59 have been exclusively by the use of anti-CD59 antibodies (19, 20). However, it remains clear that the use of these antibodies, despite showing promise, may be limited in their ability to neutralize this complement regulator.

From several recent studies, rILYd4 displays promising CD59-neutralization activity (8, 27). Indeed, CDC levels are greatly increased upon rILYd4 treatment of a wide variety of cancer cell lines as well as HIV-infected T cells (8, 27). Our goal in this study was to determine the mechanism by which rILYd4 neutralizes CD59, and to elucidate whether it is exclusively through steric interference of CD59/complement binding, or whether rILYd4 affects the PM expression and/or subcellular itinerary of CD59.

Our data support a model (Fig. 12) in which the intracellular sorting and trafficking of rILYd4-CD59 complexes differs from that of anti-CD59-CD59 complexes. Although we cannot rule out distinct modes of internalization, the association of rILYd4 with CD59 leads to the post-endocytosis sorting of CD59 into lysosomes and rapid degradation within 15 min. In contrast, upon anti-CD59 antibody incubation, much of the internalized CD59 is trafficked through a well studied endocytic pathway that leads to high levels of CD59 recycling (Fig. 12) and constitutive shedding.7

Based on our experimentation, the most extensive CD59 loss induced by rILYd4 occurs via lysosomal degradation and several lines of evidence support this notion. 1) We demonstrated that in the first 15 min, rILYd4 treatment does not induce any additional shedding to the spontaneous shedding observed in the absence of rILYd4 treatment (compare lanes 4 and 3 in Fig. 7A). Addition of leupeptin (lane 5) slows CD59 shedding, as previously reported by Perez-Torres et al. (45) for EGF receptor. Hence, CD59 shedding appears to be leupeptin-sensitive, but rILYd4 does not alter the amount of shed CD59. 2) Increased co-localization of rILYd4-CD59 with LAMP1 upon leupeptin treatment (quantified in Fig. 7, B–D) directly places rILYd4-CD59 complexes in lysosomes during the first 15 min. 3) Even at later time points (i.e. at 80 min chase), CD59 shedding remained unaffected by rILYd4 (Fig. 7E).

However, although the complex localized to lysosomes (especially upon leupeptin treatment which also inhibited its degradation), we cannot rule out the possibility of additional mechanisms of degradation, including proteolysis within peripheral endosomal compartments. Although CD59 shedding was not affected by rILYd4, it remains possible that some rILYd4-bound surface-CD59 might become prone to proteolysis at the PM by proteases that reside at the cell surface. Enzymatic modification at the PM is a well established phenomenon. Moreover, glycosylation of surface proteins (i.e. glycosylation of CD59) can render some protection from such proteolysis (46, 47). If rILYd4 binding alters CD59 in a way that exposes CD59 to PM-proteases and increases its susceptibility to in situ proteolysis, then that could account for a portion of the fast disappearance of CD59 after 15 min of incubation. Because leupeptin incompletely inhibited CD59 disappearance, such putative surface proteolysis would be leupeptin-insensitive. In this scenario, a minor degree of PM proteolysis would be difficult to detect, because digested CD59 released to the cell media might not be recognized by the anti-CD59 antibody. Although rILYd4-CD59 complexes are mostly degraded, the remaining non-degraded complexes recycle back to the PM and undergo normal shedding following cleavage by “shedases” such as GPI-PLC and GPI-PLD (32).

To the best of our knowledge, physiological conditions, which culminate in such a rapid degradation of GPI-AP, have not been previously described. Ligands such as bacterial toxins and nutrients, which bind to GPI-AP or to other raft-residing components, do not direct their receptor to such rapid degradation as observed here for rILYd4. It is tempting to speculate that this rapid degradation pathway might confer an evolutionary advantage to bacteria, such as S. intermedium, which would benefit from a fast loss of surface-CD59 upon its swift internalization, thus compromising the complement system. This mechanism was indeed described for other microorganisms such as the human immunodeficiency virus (HIV), which targets CD4 molecules as their receptor, and thus compromises the immune response. Microorganisms that down-regulate and/or evade the immune system will be better equipped to proliferate. Thus, rILYd4-induced loss of CD59 might be of profound importance to ongoing attempts to improve cancer treatments that are enhanced by CDC (19, 20).
Surprisingly, the intracellular pathway of CD59 when bound to rILYd4 is in stark contrast to its itinerary when bound at the cell surface to an antibody. In the latter case, as depicted in our model (Fig. 12), most of the internalized antibody-CD59 complex traverses the early endosomes and is recycled back to the PM via recycling endosomes. The return of CD59 to the cell surface allows it to renew its activity as a complement inhibitor and thus hinder therapeutic applications. Our finding that rILYd4 directs human CD59 to rapid and massive lysosomal degradation further promotes our understanding of the underlying mechanisms by which rILYd4 neutralizes CD59 activity. Moreover, it may also facilitate design of a better dosing regimen for potential future clinical applications of rILYd4.

Understanding the differential trafficking of rILYd4-CD59 compared with the antibody-induced route, remains an important line of investigation. It is unlikely that rILYd4 masks an active sorting motif on CD59 that may be necessary for targeting to the recycling pathway because rILYd4 binds to a region of CD59 that is directed toward the vesicular lumen. For the same reason, we view it as equally unlikely that rILYd4 itself serves as a signal for transport to lysosomes. One possibility is that the CD59 binding site for rILYd4 might be the same binding region connecting to another membrane protein, which normally regulates CD59 internalization, and rILYd4 binding may release this regulation. Our data suggest that rILYd4 (essentially a monomeric ligand), causes little or no clustering of GPI-APs and rafts at the surface, a mechanism usually considered necessary for GPI-AP internalization (35).

The “normal” entry mode and intracellular route of GPI-APs is not known, because these proteins have been studied almost exclusively in the context of ligand (or antibody) binding. Monovalent ligands for GPI-APs such as folate, do not change the diffuse distribution of the folate receptor at the PM and the ovalent ligands for GPI-APs such as folate, do not change the antilign of the receptor (36). Nevertheless, whereas most cargos of GEECs proceed toward early and recycling endosomes, in the case of the rILYd4-CD59, the complex likely does not reach early or recycling endosomes. Instead, the internalized complex is sorted differently within the cell and rapidly undergoes degradation. To our knowledge, this is the first evidence of such an itinerary for a GPI-AP.

In summary, the ability of rILYd4 to induce this rapid and massive degradation of CD59 may contribute to a prolonged blocking of human CD59 activity, possibly having a clinical potential for cancer and HIV treatment (8, 27). In parallel, the enhanced CD59 pinocytosis upon rILYd4 binding, and its subsequent swift degradation has important implications for our understanding of the trafficking of GPI-APs.

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