Direct targeting of membrane fusion by SNARE mimicry: Convergent evolution of *Legionella* effectors

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***Legionella pneumophila*, the Gram-negative pathogen causing Legionnaires' disease, infects host cells by hijacking endocytic pathways and forming a *Legionella*-containing vacuole (LCV) in which the bacteria replicate. To promote LCV expansion and prevent lysosomal targeting, effector proteins are translocated into the host cell where they alter membrane traffic. Here we show that three of these effectors [LegC2 (*Legionella* eukaryotic-like gene C2)/YlfB (yeast lethal factor B), LegC3, and LegC7/YlfA] functionally mimic glutamine (Q)-SNARE proteins. In infected cells, the three proteins selectively form complexes with the endosomal arginine (R)-SNARE vesicle-associated membrane protein 4 (VAMP4). When reconstituted in proteoliposomes, these proteins avidly fuse with liposomes containing VAMP4, resulting in a stable complex with properties resembling canonical SNARE complexes. Intriguingly, however, the LegC/SNARE hybrid complex cannot be disassembled by N-ethylmaleimide-sensitive factor. We conclude that LegCs use SNARE mimicry to divert VAMP4-containing vesicles for fusion with the LCV, thus promoting its expansion. In addition, the LegC/VAMP4 complex avoids the host's disassembly machinery, thus effectively trapping VAMP4 in an inactive state.**

Legionella pneumophila | SNAREs | NSF | membrane fusion

Legionnaires' disease in humans is caused by *Legionella pneumophila* (1), which enters human monocytes and alveolar macrophages by macropinocytosis. After endocytotic uptake, *Legionella* prevents fusion with lysosomes to escape host degradation and establishes a replication niche, called the *Legionella*-containing vacuole (LCV) (2). To achieve these goals, *Legionella* translocates effector proteins through a type IVB (Icm/Dot) secretion system into the host cytoplasm or into the LCV membrane (2–5). Around 300 *Legionella* effectors were identified by genetic or bioinformatic approaches (6–8). Whereas growth and survival of *Legionella* depends on these effector proteins, they appear to be highly redundant because as many as 71 effector-encoding genes can be deleted in a single strain that retains the ability to grow in macrophages (9).

For membrane expansion, LCVs recruit trafficking vesicles from the host cell. Mainly, these vesicles originate from trafficking vesicles shuttling between the endoplasmic reticulum (ER) and the *cis*-face of the Golgi apparatus (10), although other sources of membrane, e.g., of endosomal sources, cannot be excluded (11). Thus, *Legionella* is capable of redirecting trafficking vesicles to fuse with the LCV, but the mechanisms by which this is achieved are only slowly emerging. In eukaryotes, the specificity of membrane traffic is governed by sets of regulatory proteins, which ultimately converge to regulate vesicle fusion carried out by SNARE [soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptor] proteins. The regulatory proteins include small GTPases of the Rab and Arf families. These proteins operate as molecular switches that, once activated, recruit effector proteins from the cytoplasm to ensure fusion with the correct target by controlling SNAREs. SNAREs comprise a family of small and mostly membrane-anchored proteins (12). They are characterized by coiled-coil (CC)–forming SNARE motifs that assemble between the membranes and thus initiate fusion. SNARE motifs are classified into four subfamilies termed Qa-, Qb-, Qc-, and arginine (R)-SNAREs, with one of each required for assembly of a fusion-competent SNARE complex (13). Whereas each intracellular fusion step appears to involve its own specific set of SNARE proteins, SNAREs on their own are rather promiscuous, with members of the same subfamily being capable of substituting for each other in cells, and even more so in vitro (12).

Although the function of most *Legionella* effectors is still unknown, several of them were shown to target trafficking protein, including small GTPases such as Rab1 and Arfs, or to interfere with the formation of autophagosomes (14). Moreover, LCV formation is associated with the formation of noncognate SNARE complexes between an R-SNARE functioning in trafficking between the ER and the Golgi apparatus (mSec22b) and glutamine (Q)-SNAREs normally operating at the plasma membrane [syntaxins 2, 3, 4, and synapotosomal-associated protein (SNAP)-23] (15). Complex formation appears to be enhanced by DrrA, a *Legionella* effector that binds to the SNARE Syntaxin 3, a reaction that appears to be regulated by the small GTPase Rab1 (16).

Intriguingly, some effectors bear superficial similarity to SNAREs and thus may interfere with SNARE function. For example, the IncA effector of *Chlamydia*, was shown to interfere with SNARE assembly (17, 18). More recently, another putative *Legionella* SNARE paralog was identified by bioinformatic searches (LseA) and shown to interact with host cell SNAREs (19).

In this study, we have investigated whether three structurally related *Legionella* effectors (LegC2/YlfB, LegC3, and LegC7/YlfA) (20) interact with mammalian SNAREs, and if so, whether this interaction affects SNARE function. These LegC-proteins

Significance

***Legionella pneumophila*, the bacterium causing Legionnaires' disease, invades lung cells by creating intracellular vacuoles where it is protected and where it can multiply. For expansion, the vacuoles must hijack intracellular vesicles while avoiding destruction by lysosomes. To achieve these goals, *Legionella* exports effector proteins into the cytoplasm and into the membrane of the vacuole. Here we report that certain *Legionella* effectors mimic intracellular fusion proteins of the SNARE family, allowing them to selectively fuse with specific intracellular transport vesicles, thus providing more space for the dividing bacteria. In contrast to SNAREs, however, the *Legionella* proteins can be used only once, thus allowing the bacteria to control the amount of hijacked vesicles.**

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comprise a group of transmembrane proteins that possess coiled-coil motifs reminiscent of SNARE proteins, raising the possibility that they may form hybrid complexes with endogenous SNARE proteins. Indeed, LegC3 was shown previously to inhibit SNARE-mediated homotypic fusion of yeast vacuoles in vitro, but a direct interaction between LegC3 and SNAREs was not observed (21). We found that all three LegC proteins selectively interact with the R-SNARE vesicle-associated membrane protein 4 (VAMP4), and furthermore, that the availability of VAMP4 is rate limiting for the intracellular proliferation of *Legionella*. Using in vitro fusion assays, we also report that the three LegC proteins together are capable of substituting for the endogenous Q-SNARE partners of VAMP4, resulting in the formation of hybrid LegC/SNARE complexes and fusion with an efficiency comparable to that observed with the endogenous SNARE partners of VAMP4. Intriguingly, the hybrid LegC/VAMP4 complex formed during fusion cannot be dissociated by the SNARE disassembly enzyme NSF.

Results

LegC2, LegC3, and LegC7 Resemble Mammalian Q-SNAREs and Interact with Host VAMP4. LegC proteins contain one (LegC7) or two (LegC2 and LegC3) predicted transmembrane domains (TMDs) and two predicted coiled-coil motifs of which one is reminiscent of SNARE motifs (Fig. 1*A*). Alignment with SNARE motifs from mammalian Q-SNAREs revealed the typical heptad repeat structure, with hydrophobic residues in the "a" and "d" positions, and a conserved glutamine (Q) residue in the center of the motifs, just as in the "0" layer of Q-SNAREs (13) (Fig. 1*B*). The similarities appear to be higher than in IncA, which contains helix-breaking proline residues in the C-terminal part (Fig. 1*B*). Furthermore LegC2 and LegC3 with two consecutive TMDs resemble the unique hairpin-type autophagosomal Qa-SNARE–Stx17 (22). Hence, we investigated whether the *Legionella* effector proteins LegC2/YlfB, LegC3, and LegC7/YlfA interact with host cell SNAREs to modulate membrane fusion. To examine whether LegC proteins interact with host cell R-SNAREs, we infected phorbol 12-myristate 13-acetate (PMA)-differentiated THP-1 macrophage-like cells with *Legionella* strains overexpressing either β-lactamase (TEM) or N-terminally TEM-tagged versions of one of each of the effector proteins LegC2, LegC3, or LegC7. Six hours after infection, LegC proteins were immunoprecipitated from cell lysates using anti-TEM antibodies. Immunoblotting for TEM revealed bands of the expected size in all three cases both in the lysates and immunoprecipitates [TEM (30 kDa), TEM-LegC2 (76 kDa), TEM-LegC3 (93 kDa), and TEM-LegC7 (78 kDa)]. We then examined the precipitated material for the presence of mammalian R-SNAREs including VAMP2/Synaptobrevin and VAMP3/Cellubrevin (exocytosis) (23), VAMP4 (*trans*-Golgi network) (24), VAMP8 (late endosome/lysosome), as well as Sec22b and Ykt6 (ER–Golgi transport) (25). Whereas all six R-SNARE proteins were clearly detectable in the lysate, VAMP4 was the only R-SNARE that selectively coprecipitated with each of the tagged LegC proteins (Fig. 1*C*). Conversely, immunoprecipitations using anti-VAMP4 antibodies resulted in the coprecipitation of TEM-fused LegC2, LegC3, and LegC7, confirming that the interaction is specific (Fig. S1).

VAMP4 Knockdown in THP-1 Cells Reduces Intracellular Growth of *Legionella*. Next we asked whether VAMP4 is required for infection and/or intracellular proliferation of *Legionella*. We generated three different THP-1 cell lines in which VAMP4 expression is stably knocked down (Fig. 2*A*), whereas cell viability remains unaffected (Fig. S2). Intracellular growth of *Legionella* was monitored by fluorescence increase after infection (26) with a

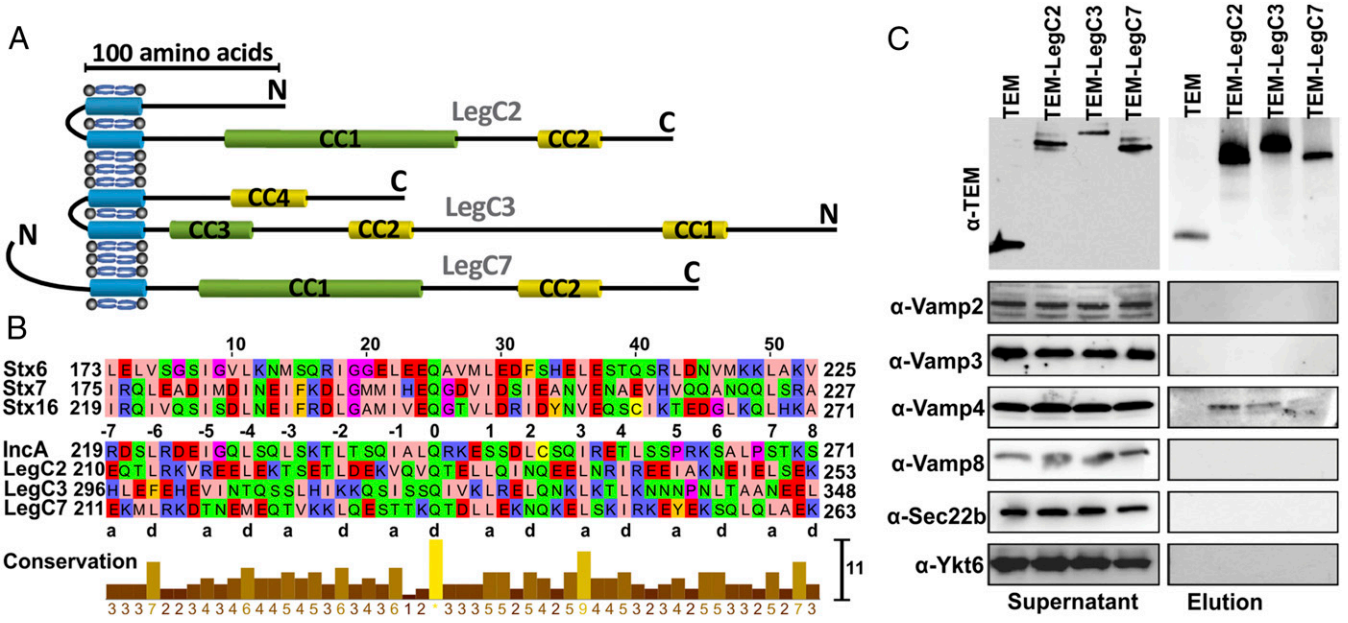

Fig. 1. LegC2, LegC3, and LegC7 resemble mammalian Q-SNAREs and interact with host VAMP4. (*A*) Predicted TMDs and the CC motifs in LegC proteins. CC motifs with probability scores <99% are shown in yellow and those with probability scores >99% are shown in green. (*B*) Multiple sequence alignments of the high-score CC motifs (green) of LegC effectors with the SNARE motifs of the endosomal Q-SNAREs Stx6 (173–225), Stx7 (175–227), and Stx16 (219–271). The hydrophobic residues in positions "a" and "d" of the heptad-repeats are indicated. LegC2, LegC3, and LegC7 also contain the conserved Q residue as in the central 0 layer of Q-SNARE. In comparison, the predicted CC motif of IncA (219–271) shows lower similarities in the C-terminal region. (*C*) VAMP4 but no other R-SNARE coprecipitates with LegC2, LegC3, and LegC7, respectively. Differentiated THP-1 cells were infected with *Legionella* strains either overexpressing TEM or individual N-terminal TEM-fused effector proteins for 6 h. Cells were lysed and supernatants were precleared with protein A/G agarose, followed by incubation with protein A/G agarose containing purified anti-TEM antibodies. The beads were washed and eluted at low pH buffer. The precleared supernatants and elutions from coimmunoprecipitation experiments were analyzed by Western blotting using anti-TEM, anti-VAMP2, anti-VAMP3, anti-VAMP4, anti-VAMP8, anti-Sec22b, or anti-Ykt6 antibodies, respectively. Of the six targeted R-SNAREs, only VAMP4 coprecipitates with the LegC proteins.

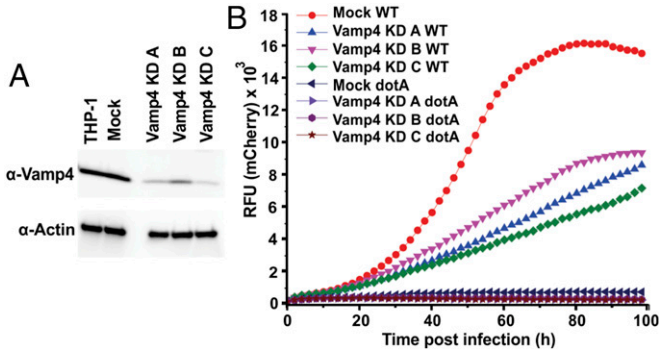

Fig. 2. Decreased expression of VAMP4 in THP-1 cells reduces intracellular growth of *Legionella*. (*A*) Immunoblot of lysates from THP-1 macrophage cell lines showing significant reduction in levels of VAMP4 with the three different knockdown constructs KD_A, KD_B, and KD_C in comparison with mock and negative control. (*B*) Intracellular growth of *Legionella* is reduced in cells in which VAMP4 expression is knocked down. For monitoring growth, a wild-type *Legionella* strain expressing mCherry as fluorescent marker was used, allowing for quantification of *Legionella* using fluorescence intensity. As controls, cells were also infected with a *dotA* mutant of *Legionella*, which fails to grow in both mock and knockdown constructs. The *Legionella* growth in VAMP4 KD cell lines were repeated in at least three independent experiments with similar results. The graph shows the result of one representative independent experiment. Each data point represents the average of technical triplicates. The nonparametric *t* test was performed between the indicated KD cell lines with the mock cell line infected with wild-type *Legionella*. The following *P* values were obtained comparing the mock-KD cells to the VAMP4 KD cell lines: VAMP4_KD_A ($P = 0.0007$); VAMP4_KD_B ($P = 0.0043$); and VAMP4_KD_C ($P < 0.0001$). All of the *P* values in these *t* tests are <0.01. Thus, the *Legionella* growth in Vamp4_KD A, B, and C are statistically different from wild-type *Legionella* growth in mock cell line at *P* value of 0.01.

mCherry-expressing wild-type strain (27). Growth was significantly reduced in all three knockdown cell lines (Fig. 2*B*). As a control, we infected the cells with a *Legionella dotA* mutant that fails to grow intracellularly (2) and found negligible growth for both mock and knockdown constructs (Fig. 2*B*). In contrast, no differences were observed in pathogen uptake (Fig. S3), showing that VAMP4 is not rate limiting for phagocytosis, but rather for intracellular growth.

LegCs Mediate Membrane Fusion Along with Host R-SNAREs and Form SNARE-Like, SDS-Resistant Complexes. We then asked whether the LegC proteins can functionally substitute for Q-SNAREs by mediating fusion with membranes containing VAMP4. To this end, we reconstituted purified VAMP4 and the three LegC proteins in small unilamellar vesicles (SUVs), respectively. Using a standard FRET assay (28, 29) robust fusion, comparable to that of vesicles containing the canonical Q-SNAREs Syntaxin 13, Syntaxin 6, and VTI1a, was observed (Fig. 3). Because SNAREs show promiscuity (12) both in vivo (30–34) and in vitro (35, 36), we replaced the R-SNARE VAMP4 with VAMP2 and still observed fusion (Fig. 3). We have observed SNARE promiscuity in fusion assays earlier as well (37). The fusions could be completely blocked by competitive inhibition (29) using the cytoplasmic domains of either VAMP4 or VAMP2 (Fig. 3). To test whether LegC effectors can mediate membrane fusion in other topologies, we reconstituted them on liposomes together with VAMP4 in 12 different combinations. Fusion was monitored by the same FRET-based assay (Fig. S4). Fusion was observed only when proteoliposomes reconstituted with LegC2, LegC3, and LegC7 were mixed with VAMP4 reconstituted proteoliposomes. Hence, no fusion was observed if one of the LegC proteins was omitted or if various combinations of LegC proteins were used in the absence of the R-SNARE (Fig. S4).

To test whether the LegC proteins form hybrid complexes with VAMP4, mixtures of purified LegC2, LegC3, and LegC7 were incubated overnight at 4 °C in the presence or absence of purified VAMP4 (Fig. 4*A*) and then resolved by SDS/PAGE without heat denaturation of the samples, followed by Coomassie staining (Fig. 4*B*). This procedure has shown previously to preserve, at least in part, SNARE complexes that then migrate with higher M_r during SDS/PAGE (38). A band of lower mobility was visible only when VAMP4 was present during the incubation (Fig. 4*B*). This band was cut out and analyzed by mass spectrometry. The resulting peptide hits were searched against a local database comprising the proteins LegC2, LegC3, LegC7, and VAMP4 using Mascot (Matrix Science). This led to unequivocal identification of all four proteins as indicated (Fig. 4*C*) by the percentage coverage (which is the percentage of all of the amino acids for a given protein that were detected in the sample). Together, these data show that LegC2, LegC3, and LegC7 form a SDS-resistant complex with VAMP4.

Unlike SNARE Complexes, the LegC–R-SNARE Complex Is Resistant to NSF/α-SNAP–Mediated Disassembly. Endogenous SNARE complexes are disassembled by the AAA^+ ATPase NSF, which regenerates fusion-competent SNAREs (12). For disassembly, the cofactor α-SNAP first binds to SNARE complexes, followed by the recruitment of NSF (39). To test whether the LegC–R-SNARE hybrids can be disassembled, we reconstituted a complex comprising LegC2, LegC3, LegC7, and Oregon Green-labeled cytoplasmic domain of Synaptobrevin (VAMP2*) (40) on SUVs and measured mobility changes associated with protein binding and complex disassembly by fluorescence anisotropy. A truncated neuronal SNARE ternary complex (41) comprising the same VAMP2*, Syntaxin 1 (183–288), and SNAP-25 (1–206) was reconstituted on SUVs (42), as positive control. Addition of α-SNAP caused increase in anisotropy due to its binding to the SNARE complex, followed by a decrease in anisotropy upon addition of NSF due to the dissociation of the VAMP2* (Fig. 5*A*). In contrast, adding α-SNAP and NSF sequentially to the LegC–VAMP2* hybrid complex did not change anisotropy, suggesting that the complex was not recognized by α-SNAP. As an additional control, the proteoliposomes were incubated with α-SNAP and

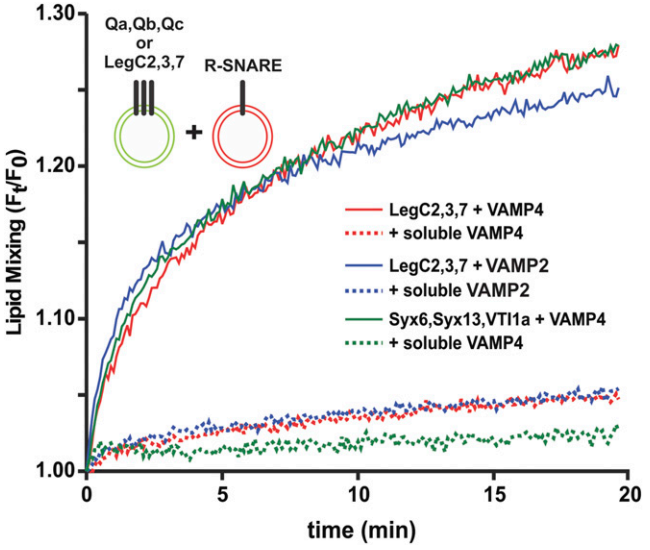

Fig. 3. Reconstitution of fusion by LegCs and host R-SNAREs. SUVs reconstituted with LegC2, LegC3, and LegC7 fuse with SUVs reconstituted with either VAMP4 (red) or VAMP2 (blue). The fusion rate was comparable to that observed with the endogenous endosomal Qa, Qb, and Qc SNAREs (green). Fusion was monitored as FRET based on lipid-mixing assay and all fusion reactions were inhibited by the soluble domains (20) of the corresponding R-SNAREs (dotted traces).

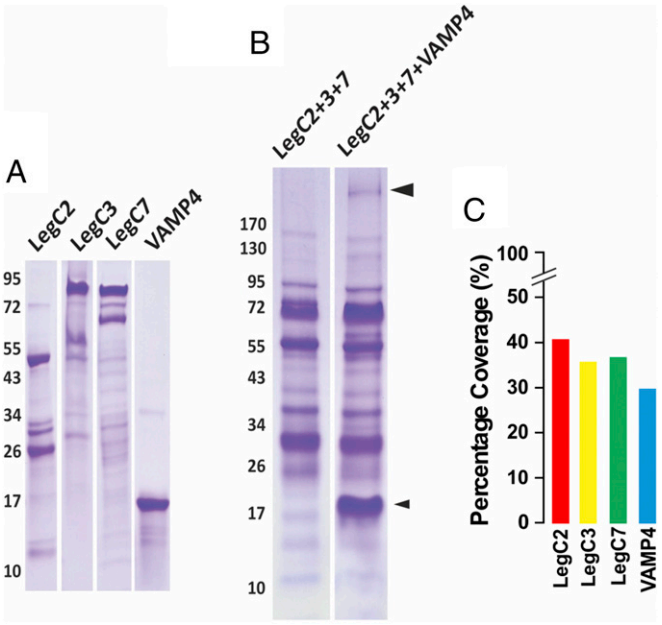

Fig. 4. LegC2, LegC3, LegC7, and VAMP4 form SDS-resistant complexes. (*A*) Relative purity of the recombinant, purified LegC2, LegC3, LegC7, and VAMP4 proteins used in this study, illustrated as Coomassie-stained bands upon SDS/PAGE. Note that the strongly stained upper bands correspond to the expected M_r of the respective proteins. (*B*) Purified LegC2, LegC3, and LegC7 were incubated with or without VAMP4, followed by SDS/PAGE and Coomassie staining. The lower (smaller) arrowhead indicates VAMP4; the upper (bigger) arrowhead indicates the LegC–VAMP4 hybrid complex (band of slower mobility). (*C*) Percentage coverage of all four proteins derived from proteomic analysis of the latter band.

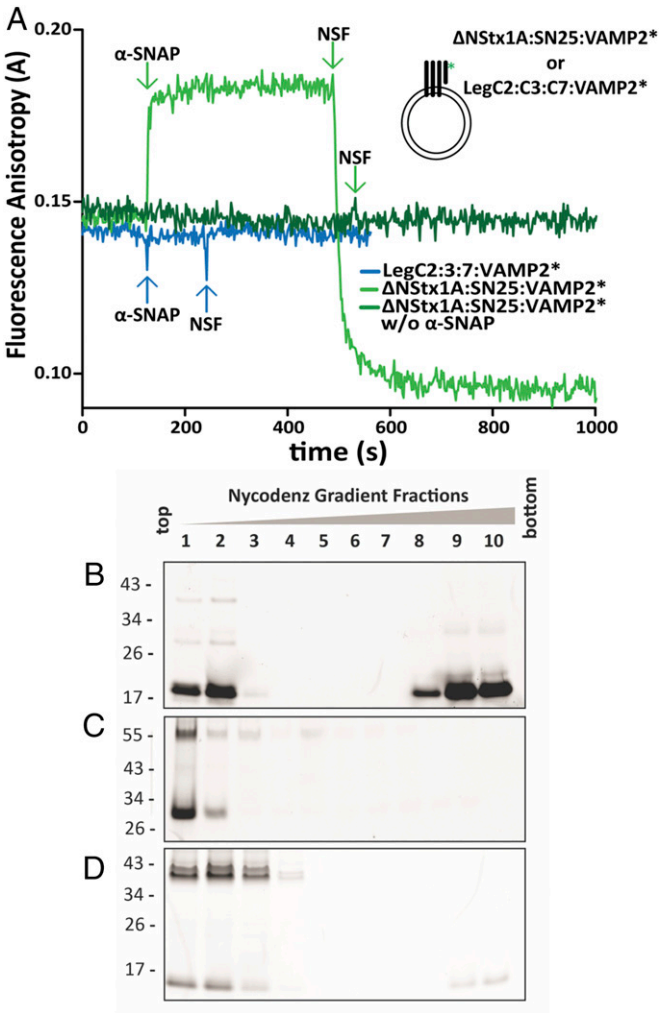

Fig. 5. LegC–R-SNARE complexes are resistant to NSF-mediated disassembly. (*A*) Binding of α-SNAP and disassembly by NSF monitored by fluorescence anisotropy of labeled, cytoplasmic Syb (VAMP2*) reconstituted on SUVs with neuronal Q-SNAREs or LegCs. As positive control, sequential addition of α-SNAP (500 nM) and NSF (60 nM) to the truncated, ternary neuronal SNARE complex, led to expected rise and fall in anisotropy, respectively (light green trace). In the absence of α-SNAP, the same complex showed no change in anisotropy upon NSF addition (dark green trace). There was no change in anisotropy upon addition of α-SNAP and NSF to the LegC–VAMP2* hybrid complex, indicating that α-SNAP does not bind the complex, preventing binding of NSF (blue trace). For all SUVs, the protein:lipid (molar ratio) was 1:1,000. For purity of the recombinant proteins see Fig. 4*A*. (*B–D*) Proteoliposomes (as in *A*) reconstituted with truncated ternary neuronal SNARE complexes or LegC–VAMP2* hybrid complexes were incubated with α-SNAP, NSF, Mg^{+2}, and ATP for 10 min followed by loading the samples on the bottom of a Nycodenz density gradient. After ultracentrifugation, the liposomes are enriched in the *Top* fractions of the gradient, whereas proteins not associated with membranes remain at the *Bottom*. The fractions were resolved by SDS/PAGE and the Oregon-Green–labeled, cytoplasmic Syb (VAMP2*) was detected in gel by a fluorescence imager. (*B*) In proteoliposomes containing the neuronal SNARE complexes, the majority of the VAMP2* was released and remained at the bottom of the gradient. Part of the unassembled VAMP2* seen in the *Upper* fractions illustrates the successful reconstitution of the complex on liposomes. (*C*) Identical to *B*, but ATP was replaced with its analog ATPγS. No dissociation of VAMP2* was detectable. Furthermore an SDS-resistant band of approximately 55 kDa is visible as expected for a truncated ternary neuronal SNARE complex. (*D*) For proteoliposomes containing the LegC–VAMP2 hybrid complexes, VAMP2* was not released (note that the weak signal on the bottom of the gradient represent the excess VAMP2* that did not form the complex). In addition, prominent SDS-resistant bands of higher M_r are detectable, representing nondissociated complexes.

NSF in the presence of Mg^{+2} and ATP for 10 min followed by flotation gradients to separate liposomes and soluble proteins (Fig. 5*B*). In the case of proteoliposomes reconstituted with the neuronal SNARE complex, the majority of the VAMP2* was detected in the soluble fraction, whereas in the case of the LegC–VAMP2* proteoliposomes, the VAMP2* remained in the liposomal fraction, confirming that NSF is unable to disassemble the LegC–R-SNARE hybrid complexes.

Discussion

In this study, we have shown that the three *Legionella* effectors LegC2, LegC3, and LegC7 together form fully functional SNARE acceptor complexes that mediate membrane fusion by forming SNARE-like hybrid complexes with R-SNAREs. We have also shown that, although promiscuous in vitro, the LegC proteins selectively bind to the R-SNARE VAMP4, and that VAMP4 is required for supporting the growth of *Legionella* in infected cells.

Our findings provide a fascinating example of molecular mimicry by convergent evolution. Despite the similarities between LegC coiled-coil domains and SNARE motifs (Fig. 1), none of the LegC domains is significantly homologous (43) to SNAREs, indicating that they are not evolutionarily related. Due to this lack of homology, SNARE mimicry is difficult to distinguish from functionally unrelated coiled-coil motifs using bioinformatics approaches.

The capability of fully substituting for the endogenous SNAREs (at least in vitro) is unexpected and highly surprising. Whereas there is some variability in SNARE structure, the TMDs of SNAREs are typically at the C-terminal end. More importantly, they are separated from the interacting SNARE motifs only by few amino acids, with the distance between membrane anchor and SNARE motifs thought to be critical for their ability to transmit energy from the zippering to the fusion reaction (44). Whereas the

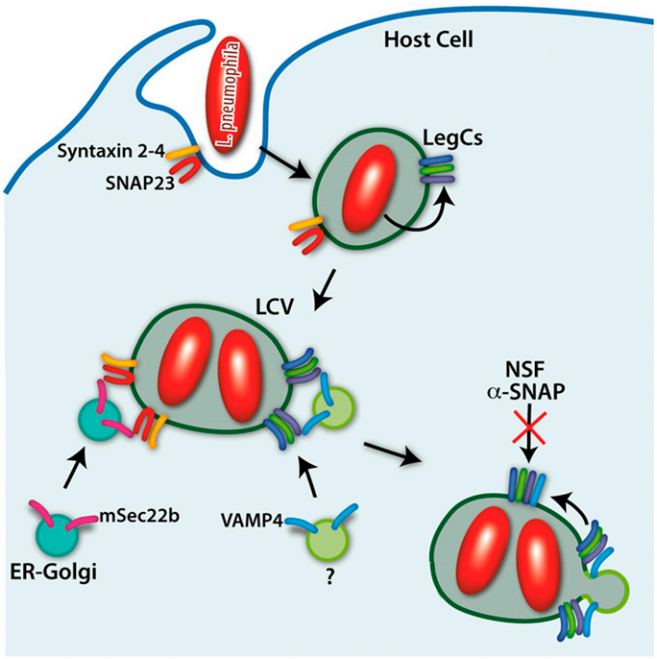

Fig. 6. Model showing manipulation of membrane traffic by *Legionella* LegC proteins using SNARE mimicry. LegC proteins on the LCV membrane recruit VAMP4 vesicles (among others) to acquire membranes for vacuolar growth to facilitate proliferation of the pathogen.

overall domain structure of the LegC proteins resembles many SNAREs (TMD connected by a linker to the coiled-coil motif), linker length in all cases is substantially higher than in SNAREs. Even more surprisingly, in both LegC2 and LegC7, the orientation of the coiled-coil motifs is inverted, which may put constraints on the alignment with SNAREs (parallel or antiparallel). Presently we do not know yet in which way the proteins interact which each other, which of the coiled-coils are participating, and to what extent the hybrid complexes are structurally similar to the highly conserved SNARE four-helix bundles. However, it appears that the structural tolerance of the SNARE-based zipper mechanism for membrane fusion is much higher than anticipated (45), which is also supported by the surprising structural diversity of artificial SNARE mimetics capable of inducing fusion in vitro by some kind of zippering mechanism (46, 47). Further structural investigation of the LegC/SNARE hybrid complex can thus be expected to yield novel insights not only into the degree of structural conservation but also into the mechanisms involved in SNARE-mediated membrane fusion.

The ability of *Legionella* to use the R-SNARE VAMP4 as a "one-shot" device, enabling fusion with vesicles needed for expansion of the LCV, but then preventing the reactivation of the LegC/SNARE hybrid complex by blocking its disassembly, leads to an irreversible hijacking of the SNARE machinery by the pathogen. This is a fascinating novel example of the emerging arsenal used by intracellular pathogens for manipulating host cells. At present the contribution of this pathway to the overall growth of the LCV in comparison with the pathway involving ER-derived trafficking vesicles is difficult to evaluate (Fig. 6). It is also possible that other effectors are functionally equivalent to the LegC proteins studied here. Taken together, the ability of pathogen effectors to form coiled-coil complexes with host SNAREs that not only inhibit but also functionally substitute for endogenous SNAREs constitutes yet another mechanism by which intracellular pathogens manipulate the membrane traffic of host cells for survival and growth.

Materials and Methods

Detailed protocols for all sections are described in *SI Materials and Methods*.

Bacterial Strains, Plasmids, and Oligonucleotides. *Legionella* strains are all derivatives of *L. pneumophila*, Philadelphia-1, and their construction is described in detail in *SI Materials and Methods*. Oligonucleotide sequences used to amplify relevant *Legionella* genes are listed in *SI Materials and Methods*. The resulting plasmid constructs are also described in *SI Materials and Methods*.

Cell Culture. THP-1 cells were obtained from American Type Culture Collection and grown in Advanced RPMI 1640 (Invitrogen) supplemented with 10% (vol/vol) FBS and 2 mM glutamine at 37 °C in a CO_2 incubator. THP-1 cells were differentiated into macrophage-like cells by resuspending them into RPMI + 2 mM glutamine + 10% (vol/vol) FBS + 30 μM PMA. Following 72 h of treatment with PMA, the differentiated THP-1 cells were washed and resuspended in RPMI + 2 mM glutamine + 10% (vol/vol) FBS for infection (20).

Infection of THP-1 Cells by *Legionella* and Coimmunoprecipitation of LegC Proteins and VAMP4. Coimmunoprecipitation experiments were carried following the manual of the Pierce Crosslink IP Kit (Thermo Fisher Scientific); additional details are described in *SI Materials and Methods*.

VAMP4 Knockdown. Expression of VAMP4 in THP-1 cells was decreased by creating stable cell lines that express a shRNA complementary to the VAMP4 transcript carried on a lentivirus vector as detailed in *SI Materials and Methods*.

Protein Purification. Proteins were overexpressed as N-terminal 6×-His tagged recombinant polypeptides using pET15b vector (Novagen) in the *Escherichia coli* strain BL21 (DE3) and affinity purified using Ni^{2+}-nitrilotriacetic acid (NTA) agarose (Qiagen) resin followed by ion-exchange chromatography using the ÄKTA system (GE Healthcare) as described earlier (37, 39). The truncated, ternary neuronal SNARE complex was formed by mixing Syntaxin 1A (183–288), SNAP-25 (1–206), and Oregon-Green–labeled (at position S28C) cytoplasmic domain (1–96) of Syb (VAMP2*), in the molar ratio = 1:1:1.5 and purified as described previously (41); additional details are described in *SI Materials and Methods*.

Proteoliposome Preparation and Fusion Assay. Proteoliposomes were prepared by reconstituting the proteins and lipids (both dissolved in detergents) into SUVs by detergent removal through a Sephadex G-50 column. Fusion of liposomes was monitored by lipid mixing assay based on FRET using a spectrofluorimeter (FluoroMax-2; Jobin Yvon) and NSF-mediated disassembly was monitored by fluorescence anisotropy using a FluoroLog 3 spectrometer in a T configuration equipped for polarizers (model FL322; Jobin Yvon); additional details are described in *SI Materials and Methods*.

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1. McDade JE, et al. (1977) Legionnaires' disease: Isolation of a bacterium and demonstration of its role in other respiratory disease. *N Engl J Med* 297(22):1197–1203.
2. Hubber A, Roy CR (2010) Modulation of host cell function by *Legionella pneumophila* type IV effectors. *Annu Rev Cell Dev Biol* 26:261–283.
3. Berger KH, Isberg RR (1993) Two distinct defects in intracellular growth complemented by a single genetic locus in *Legionella pneumophila*. *Mol Microbiol* 7(1):7–19.
4. Segal G, Purcell M, Shuman HA (1998) Host cell killing and bacterial conjugation require overlapping sets of genes within a 22-kb region of the *Legionella pneumophila* genome. *Proc Natl Acad Sci USA* 95(4):1669–1674.
5. Vogel JP, Andrews HL, Wong SK, Isberg RR (1998) Conjugative transfer by the virulence system of *Legionella pneumophila*. *Science* 279(5352):873–876.
6. Isaac DT, Isberg R (2014) Master manipulators: An update on *Legionella pneumophila* Icm/Dot translocated substrates and their host targets. *Future Microbiol* 9(3):343–359.
7. Franco IS, Shuman HA, Charpentier X (2009) The perplexing functions and surprising origins of *Legionella pneumophila* type IV secretion effectors. *Cell Microbiol* 11(10):1435–1443.
8. Ensminger AW (2016) *Legionella pneumophila*, armed to the hilt: Justifying the largest arsenal of effectors in the bacterial world. *Curr Opin Microbiol* 29:74–80.

9. O'Connor TJ, Adepoju Y, Boyd D, Isberg RR (2011) Minimization of the *Legionella pneumophila* genome reveals chromosomal regions involved in host range expansion. *Proc Natl Acad Sci USA* 108(36):14733–14740.
10. Dorer MS, Kirton D, Bader JS, Isberg RR (2006) RNA interference analysis of *Legionella* in *Drosophila* cells: Exploitation of early secretory apparatus dynamics. *PLoS Pathog* 2(4):e34.
11. Personnic N, Bärlocher K, Finsel I, Hilbi H (2016) Subversion of retrograde trafficking by translocated pathogen effectors. *Trends Microbiol* 24(6):450–462.
12. Jahn R, Scheller RH (2006) SNAREs: Engines for membrane fusion. *Nat Rev Mol Cell Biol* 7(9):631–643.
13. Fasshauer D, Sutton RB, Brunger AT, Jahn R (1998) Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNAREs. *Proc Natl Acad Sci USA* 95(26):15781–15786.
14. Choy A, et al. (2012) The *Legionella* effector RavZ inhibits host autophagy through irreversible Atg8 deconjugation. *Science* 338(6110):1072–1076.
15. Arasaki K, Roy CR (2010) *Legionella pneumophila* promotes functional interactions between plasma membrane syntaxins and Sec22b. *Traffic* 11(5):587–600.
16. Arasaki K, Toomre DK, Roy CR (2012) The *Legionella pneumophila* effector DrrA is sufficient to stimulate SNARE-dependent membrane fusion. *Cell Host Microbe* 11(1): 46–57.
17. Delevoye C, et al. (2008) SNARE protein mimicry by an intracellular bacterium. *PLoS Pathog* 4(3):e1000022.
18. Paumet F, et al. (2009) Intracellular bacteria encode inhibitory SNARE-like proteins. *PLoS One* 4(10):e7375.
19. King NP, et al. (2015) Soluble NSF attachment protein receptor molecular mimicry by a *Legionella pneumophila* Dot/Icm effector. *Cell Microbiol* 17(6):767–784.
20. de Felipe KS, et al. (2008) *Legionella* eukaryotic-like type IV substrates interfere with organelle trafficking. *PLoS Pathog* 4(8):e1000117.
21. Bennett TL, et al. (2013) LegC3, an effector protein from *Legionella pneumophila*, inhibits homotypic yeast vacuole fusion in vivo and in vitro. *PLoS One* 8(2):e56798.
22. Itakura E, Kishi-Itakura C, Mizushima N (2012) The hairpin-type tail-anchored SNARE syntaxin 17 targets to autophagosomes for fusion with endosomes/lysosomes. *Cell* 151(6):1256–1269.
23. Calakos N, Bennett MK, Peterson KE, Scheller RH (1994) Protein-protein interactions contributing to the specificity of intracellular vesicular trafficking. *Science* 263(5150): 1146–1149.
24. Steegmaier M, Klumperman J, Foletti DL, Yoo JS, Scheller RH (1999) Vesicle-associated membrane protein 4 is implicated in *trans*-Golgi network vesicle trafficking. *Mol Biol Cell* 10(6):1957–1972.
25. Zhang T, Hong W (2001) Ykt6 forms a SNARE complex with syntaxin 5, GS28, and Bet1 and participates in a late stage in endoplasmic reticulum-Golgi transport. *J Biol Chem* 276(29):27480–27487.
26. Levi A, Folcher M, Jenal U, Shuman HA (2011) Cyclic diguanylate signaling proteins control intracellular growth of *Legionella pneumophila*. *MBio* 2(1):e00316–e10.
27. Charpentier X, et al. (2009) Chemical genetics reveals bacterial and host cell functions critical for type IV effector translocation by *Legionella pneumophila*. *PLoS Pathog* 5(7):e1000501.
28. Struck DK, Hoekstra D, Pagano RE (1981) Use of resonance energy transfer to monitor membrane fusion. *Biochemistry* 20(14):4093–4099.
29. van den Bogaart G, et al. (2010) One SNARE complex is sufficient for membrane fusion. *Nat Struct Mol Biol* 17(3):358–364.
30. Tsui MM, Banfield DK (2000) Yeast Golgi SNARE interactions are promiscuous. *J Cell Sci* 113(Pt 1):145–152.
31. Wendler F, Tooze S (2001) Syntaxin 6: The promiscuous behaviour of a SNARE protein. *Traffic* 2(9):606–611.
32. Hohenstein AC, Roche PA (2001) SNAP-29 is a promiscuous syntaxin-binding SNARE. *Biochem Biophys Res Commun* 285(2):167–171.
33. Kweon Y, Rothe A, Conibear E, Stevens TH (2003) Ykt6p is a multifunctional yeast R-SNARE that is required for multiple membrane transport pathways to the vacuole. *Mol Biol Cell* 14(5):1868–1881.
34. Bethani I, et al. (2007) The specificity of SNARE pairing in biological membranes is mediated by both proof-reading and spatial segregation. *EMBO J* 26(17):3981–3992.
35. Yang B, et al. (1999) SNARE interactions are not selective. Implications for membrane fusion specificity. *J Biol Chem* 274(9):5649–5653.
36. Fasshauer D, Antonin W, Margittai M, Pabst S, Jahn R (1999) Mixed and non-cognate SNARE complexes. Characterization of assembly and biophysical properties. *J Biol Chem* 274(22):15440–15446.
37. Brandhorst D, et al. (2006) Homotypic fusion of early endosomes: SNAREs do not determine fusion specificity. *Proc Natl Acad Sci USA* 103(8):2701–2706.
38. Hayashi T, et al. (1994) Synaptic vesicle membrane fusion complex: Action of clostridial neurotoxins on assembly. *EMBO J* 13(21):5051–5061.
39. Winter U, Chen X, Fasshauer D (2009) A conserved membrane attachment site in alpha-SNAP facilitates N-ethylmaleimide-sensitive factor (NSF)-driven SNARE complex disassembly. *J Biol Chem* 284(46):31817–31826.
40. Fasshauer D, Eliason WK, Brünger AT, Jahn R (1998) Identification of a minimal core of the synaptic SNARE complex sufficient for reversible assembly and disassembly. *Biochemistry* 37(29):10354–10362.
41. Pobbati AV, Stein A, Fasshauer D (2006) N- to C-terminal SNARE complex assembly promotes rapid membrane fusion. *Science* 313(5787):673–676.
42. Schuette CG, et al. (2004) Determinants of liposome fusion mediated by synaptic SNARE proteins. *Proc Natl Acad Sci USA* 101(9):2858–2863.
43. Kloepper TH, Kienle CN, Fasshauer D (2007) An elaborate classification of SNARE proteins sheds light on the conservation of the eukaryotic endomembrane system. *Mol Biol Cell* 18(9):3463–3471.
44. Chernomordik LV, Zimmerberg J, Kozlov MM (2006) Membranes of the world unite! *J Cell Biol* 175(2):201–207.
45. Jahn R, Fasshauer D (2012) Molecular machines governing exocytosis of synaptic vesicles. *Nature* 490(7419):201–207.
46. Chan YH, van Lengerich B, Boxer SG (2009) Effects of linker sequences on vesicle fusion mediated by lipid-anchored DNA oligonucleotides. *Proc Natl Acad Sci USA* 106(4):979–984.
47. Lygina AS, Meyenberg K, Jahn R, Diederichsen U (2011) Transmembrane domain peptide/peptide nucleic acid hybrid as a model of a SNARE protein in vesicle fusion. *Angew Chem Int Ed Engl* 50(37):8597–8601.
48. Lupas A, Van Dyke M, Stock J (1991) Predicting coiled coils from protein sequences. *Science* 252(5009):1162–1164.
49. Edgar RC (2004) MUSCLE: A multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics* 5:113.
50. Waterhouse AM, Procter JB, Martin DM, Clamp M, Barton GJ (2009) Jalview Version 2: A multiple sequence alignment editor and analysis workbench. *Bioinformatics* 25(9):1189–1191.
51. Livingstone CD, Barton GJ (1993) Protein sequence alignments: A strategy for the hierarchical analysis of residue conservation. *Comput Appl Biosci* 9(6):745–756.
52. Letunic I, Doerks T, Bork P (2012) SMART 7: Recent updates to the protein domain annotation resource. *Nucleic Acids Res* 40(Database issue):D302–D305.
53. Sadosky AB, Wiater LA, Shuman HA (1993) Identification of *Legionella pneumophila* genes required for growth within and killing of human macrophages. *Infect Immun* 61(12):5361–5373.
54. Stein A, Weber G, Wahl MC, Jahn R (2009) Helical extension of the neuronal SNARE complex into the membrane. *Nature* 460(7254):525–528.
55. Antonin W, Fasshauer D, Becker S, Jahn R, Schneider TR (2002) Crystal structure of the endosomal SNARE complex reveals common structural principles of all SNAREs. *Nat Struct Biol* 9(2):107–111.
56. Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat Protoc* 1(6):2856–2860.
57. Hernandez JM, Kreutzberger AJ, Kiessling V, Tamm LK, Jahn R (2014) Variable cooperativity in SNARE-mediated membrane fusion. *Proc Natl Acad Sci USA* 111(33): 12037–12042.
58. Fiske CH, Subbarow Y (1925) The colorimetric determination of phosphorus. *J Biol Chem* 66:375–400.
59. Böttcher CJF, Pries C, van Gent CM (1961) A rapid and sensitive colorimetric microdetermination of free and bound choline. *Recl Trav Chim* 80:1169–1178.
60. Hernandez JM, et al. (2012) Membrane fusion intermediates via directional and full assembly of the SNARE complex. *Science* 336(6088):1581–1584.
61. Marra A, Horwitz MA, Shuman HA (1990) The HL-60 model for the interaction of human macrophages with the Legionnaires' disease bacterium. *J Immunol* 144(7): 2738–2744.