Perspectives Series: Host/Pathogen Interactions

Membrane-Protein Traffic in Pathogen-infected Cells

Keith A. Joiner
Section of Infectious Diseases, Yale University School of Medicine, New Haven, Connecticut 06520-8022

For more than two decades, the standard paradigm for discussing membrane traffic in pathogen-infected cells has centered around fusion of the microbe-containing vacuole with lysosomes, and upon vacuole acidification. Organisms have been placed in one of three relatively invariant categories: pathogens residing in vacuoles which fuse with lysosomes and acidify, those that readily disrupt the vacuolar membrane and subsequently replicate in the host cell cytosol, and microbes residing in vacuoles which neither fuse with lysosomes nor acidify. The nature of membrane traffic was thought to be dictated almost exclusively by the requirement of the pathogen to avoid killing by the low pH and the degradative enzymes present in lysosomes.

It is increasingly clear that this paradigm is oversimplified (1, 2). It neither appropriately reflects the selectivity and plasticity of membrane traffic within cells, nor does it give adequate weight to membrane traffic events which may be largely of importance for nutrient acquisition or induction of gene expression by the intracellular organism. This perspective will describe membrane traffic in pathogen-infected cells in the context of more recent advances in the cell biology of vesicular traffic, and with the above considerations in mind.

Phagosome biogenesis and maturation: relationship to pathogen vacuoles

Current understanding of the fate of an inert particle internalized by phagocytosis is to rapidly proceed through an extensive series of membrane fusion and budding/remodeling events which eventually deliver the particle to a phagolysosome. After internalization, modification of the phagosome membrane occurs rapidly in a manner which is dependent upon the nature of the particle itself and upon the host cell type. In phagocytic cells, plasma membrane proteins are rapidly removed, and fusion with early endosomes occurs within several minutes. Even proteins localized predominantly to late endosomes can be added to steady state within as short a period as 5 min, and can be largely removed after an additional 5–10 min of incubation. Hence, experiments which do not look at early time points may substantially oversimplify the membrane traffic events involved in phagosome maturation, whether with pathogens or with inert particles.

With the development of new integral membrane protein markers of the endocytic cascade, it is also clear that the morphology of cellular compartments is more complex than appreciated previously. For example, certain macrophages contain extensive tubuloreticular compartments bearing a high concentration of lysosome-associated membrane proteins (LAMPs).[1] Phagosomes containing inert particles in mouse peritoneal macrophages may fuse predominantly with these tubular lysosomes, rather than with high density, electron dense, mannose-6-phosphate receptor (M6PR)–negative terminal lysosomes with pH 4.5–5.0. Therefore, previous morphologic studies analyzing delivery of electron dense markers from terminal lysosomes to pathogen vacuoles may not reflect normal phagosome-lysosome traffic as determined with more recently developed reagents and approaches.

Vacuole docking and fusion: mechanisms for control of fusion of pathogen-containing vacuoles

The SNARE hypothesis is now widely recognized and serves as a useful framework for analysis of fusion events surrounding pathogen-containing vacuoles. Since this hypothesis and the data supporting it, have been reviewed extensively (3), they will be stated here in only the briefest and most general of terms. Intracellular transport vesicles contain transmembrane proteins (Vesicle SNAP receptors or V-SNARES) which bind with high affinity and specificity to a complimentary set of transmembrane proteins on the target membrane (Target SNAP receptors or T-SNARES) with which they will ultimately fuse. Cytosolic proteins (*N*-ethylmaleimide sensitive factor or NSF, and soluble NSF attachment proteins or SNAPs) associate with the SNARES to assemble a 20s complex which is necessary to trigger fusion between the vesicle and target membrane. Additional proteins are required for initial vesicle budding (Coat proteins or COPs and ADP ribosylation factors or ARFs) and for activating vesicles for subsequent docking and fusion (Rabs).

In the context of the SNARE model, pathogen vacuoles which display selective or absent fusion with host cell vesicular compartments must either contain a microbial inhibitor which prevents assembly or function of the 20s docking and fusion complex, or must lack the V-SNARES or associated components (such as Rabs) necessary for vacuole activation, docking, and fusion. Additional proteins, including annexins, host cell cytoskeletal components, and the myristoylated, alanine rich C-kinase substrate (MARCKS) are also likely to participate in vacuole biogenesis or maturation.

Address correspondence to Keith A. Joiner, M.D., LCI 808, Section of Infectious Diseases, Yale University School of Medicine, 333 Cedar Street, P.O. Box 208022, New Haven, CT 06520-8022. Phone: 203-785-4140; FAX: 203-785-3864; E-mail: Keith_Joiner@qm.yale.edu

Received for publication 27 February 1997 and accepted for publication 13 March 1997.

J. Clin. Invest.

0021-9738/97/04/1814/04 $2.00
Volume 99, Number 8, April 1997, 1814–1817

1. *Abbreviations used in this paper:* ER, endoplasmic reticulum; LAMPs, lysosome-associated membrane proteins.

Most pathogen-containing vacuoles are selectively fusogenic

The majority of intracellular pathogens resides in vacuoles which are selectively fusogenic. (It may ultimately turn out that all pathogen vacuoles fuse with some, albeit highly specific, compartment.) A smaller subset of pathogen vacuoles is either apparently incapable of fusing with any vesicular compartment, or rather promiscuously fuses with many vesicular organelles. A brief description of the most well studied organisms in each group follows.

Nonfusogenic vacuoles

Only two pathogens, *Legionella pneumophila* and *Toxoplasma gondii*, reside in vacuoles not demonstrated to fuse with any organelle of the endocytic or secretory cascade.

Toxoplasma enters cells in an active fashion, dependent upon the actin cytoskeleton of the parasite but not the host cell. In a rapid (15–20s) process, active entry of *T. gondii* excludes all proteins of host cell origin from the vacuolar membrane which surrounds the pathogen, including proteins of the SNARE family. This likely explains the failure of the *T. gondii* vacuole to fuse with any compartment in the endocytic cascade, or to acidify. In contrast, altering the route of entry, by forcing the parasite to enter via a host cell–dictated phagocytic route, results in inclusion of plasma membrane proteins in the vacuole membrane, which is in turn followed by fusion, acidification, and parasite killing.

The exclusion from the vacuolar membrane of host cell plasma membrane transporters for nutrients suggests that alternative mechanisms are required for nutrient access by the intracellular parasite. A functional pore in the vacuolar membrane, likely of parasite origin, provides access of the intracellular parasite to molecules of $< 1{,}800$ D from the host cell cytosol. Tight association of the vacuolar membrane with host cell endoplasmic reticulum (ER) and mitochondria may supply lipids and ATP, respectively, to the parasite.

L. pneumophila, on the other hand, is internalized by phagocytosis. In the one strain internalized by the unusual process of coiling phagocytosis, host cell proteins are sorted from the vacuolar membrane at the time of entry. Exclusion of these proteins is not absolute, however, and coiling phagocytosis is not necessary for formation of the replicative phagosome, which promotes microbial multiplication. Since the replicative phagosome does not fuse with any endocytic organelle of the host cell and does not acidify, it is likely that the organism produces an inhibitor of the fusion process. In addition, the replicative phagosome is surrounded by host cell ER. Induction of autophagy in the host cell, through nutrient starvation, increases the extent of ER association and increases the microbial growth rate.

Genetic strategies have identified at least 16 genes required for establishing the replicative phagosome (Isberg, R., personal communication). Mutations in any of these genes lead to fusion of the legionella vacuole with LAMP-containing compartments, and the absence of ER recruitment. The best characterized of these genes, *DotA*, encodes a multimembrane-spanning protein localized to the inner membrane. It is likely that genes involved in replicative phagosome formation encode a secretion apparatus necessary for modifying the vacuolar space and/or membrane. The genetic linkage between fusion inhibition and ER recruitment reflects the close link between membrane traffic events and mechanisms for nutrient acquisition.

Selectively fusogenic

Four well-studied organisms reside either permanently or transiently in a vacuole formed or modified by a selective fusion event.

Chlamydia. Chlamydia are internalized by conventional phagocytic and endocytic events, not apparently involving sorting within the vacuolar membrane of plasma membrane proteins from the host cell. The intracellular inclusion containing *Chlamydia spp.* neither acidifies fully, nor fuses with endocytic organelles, including lysosomes. Interestingly, purified cell walls from the infective elementary body are sufficient to inhibit fusion, likely due to protein components in the membrane. No fixed protein markers or fluid phase endocytic markers are identified in the inclusion. Thus, by these criteria, the vacuole is nonfusogenic.

The chlamydial inclusion selectively fuses, however, with sphingolipid-rich vesicles derived from the *trans*-Golgi network. This process neither disrupts nor intersects with traffic of glycoproteins within the cell. This suggests that the chlamydia vacuole can be recognized by the docking and fusion machinery for a specific subset of vesicles carrying glycolipids. Although the machinery involved in docking and fusion of such vesicles is not known, it is possible that it is dependent upon annexins, rather than SNAREs, SNAPs, and Rabs (4).

Chlamydia are energy parasites, since they are entirely dependent upon their host cell to provide ATP and other high energy metabolites. Chlamydial reticulate bodies possess an efficient translocator which takes up ATP while coupling it to the expulsion of ADP. While the inclusion membrane does not contain pores, it does contain chlamydial proteins which may function in nutrient access. Furthermore, hollow projections on the reticulate body surface apparently penetrate the inclusion membrane and may have access to the host cell cytosol, suggesting that *Chlamydiae* may scavenge ATP and other nutrients using a "soup through a straw" mechanism.

Mycobacteria. Vacuoles containing *Mycobacterium avium* and *Mycobacterium tuberculosis* are also selectively fusogenic. Like chlamydia, mycobacteria are internalized into cells via conventional phagocytosis.

The working model for the last quarter century has been that the mycobacterial vacuole is nonfusogenic. It is now clear, however, that the mycobacterial vacuole intersects extensively but selectively with the recycling endosomal apparatus, and in particular with the transferrin receptor and the transferrin recycling pathway. The vacuolar membrane surrounding the organism lacks proton ATPases from the host cell, and acidification is limited, due to failure to deliver the pump to the vacuolar membrane. In contrast, the vacuolar membrane contains low to moderate amounts of LAMPs, potentially due to direct fusion with vesicles derived from the *trans*-Golgi network.

The situation with mycobacterial phagosomes is reminiscent of the exaggerated homotypic fusion of early endosomes which is induced with transfection into cells of rab5 mutants defective in GTP hydrolysis. Of direct relevance to this point, a live nonhemolytic mutant of *Listeria monocytogenes* can recruit and retain the GTPase Rab5 on the phagosome membrane in comparison to dead *Listeria* (5). Early endosomal markers and components of the 20s fusion complex accumulate on the live but not the dead listerial phagosome membrane. Although the mechanism is not clear, this suggests a

possible strategy for pathogen vacuoles to remain locked in the stage of homotypic early endosomal fusion mediated by Rab5.

Antibody-coated mycobacteria internalized by macrophages reside in vacuoles which fuse with lysosomes and acidify, yet organisms survive and may even demonstrate enhanced replication. This belies the notion that inhibition of fusion with lysosomes is primarily for purposes of avoiding destruction. Since access to intracellular iron is critical for mycobacterial growth, a reasonable hypothesis is that freezing the mycobacterial vacuole at the early endosome stage has evolved in part to facilitate iron acquisition.

Salmonella. Membrane traffic in salmonella-infected cells is dictated by the host cell type. *Salmonella typhimurium* in epithelial cells resides in a vacuole which shows limited accessibility to fluid phase endocytic tracers, and lacks the M6PR, yet rapidly acquires LAMPs and selected lysosomal enzymes in a microtubule-independent fashion. Selective fusion of the salmonella vacuole with LAMP-rich vesicles derived from the *trans*-Golgi network may explain this process. Whether this compartment fuses with endosomes immediately after formation (e.g., the extent of selectivity in fusion) is not yet known.

In contrast, a recent series of experiments suggests that *Salmonella* within macrophages resides in a compartment which colocalizes with fluid-phase endocytic tracers, is LAMP-enriched, and is, in most respects, indistinguishable from phagolysosomes. Earlier studies concluding that salmonella vacuoles did not fuse with dense terminal lysosomes presumably reflect the difference between those compartments and LAMP-rich structures in macrophages, described above. Survival of the organism in macrophages is dependent upon acidification of the vacuole to a pH of 4.0–5.0, likely associated with the induction of genes necessary for intracellular survival and replication. While the rapidity of acidification may or may not be modulated by the organism, it is clear that any modulation of vacuole fusion with endocytic organelles in macrophages cannot be for the purposes of blocking acidification.

Trypanosoma cruzi entry into cells occurs by a novel process which involves a selective fusion event. Secretion by the parasite of an as yet unidentified factor triggers a G-protein–coupled receptor in the host cell, with activation of phospholipase C, and generation of IP3, releasing calcium from intracellular stores in the host cell ER. This induces movement to and fusion with the plasma membrane of host cell lysosomes at the site of parasite attachment. Therefore, the initial vacuole is largely lysosome derived, and the vacuolar membrane which surrounds the parasite is rich in LAMPs. The vacuolar membrane is subsequently disrupted, in a process dependent upon vacuole acidification, allowing parasite replication in the cytosol. In most respects, therefore, the previous concept that *T. cruzi* resides in the cytosol as a mechanism of escaping from fusion with lysosomes could not be more wrong.

What specific advantage the process of lysosomal fusion provides to the organism during the initial entry process is not clear. In contrast, residence within the cytosol is presumed to provide maximal access to nutrients from the host. Support for this general concept comes from observations with two other organisms which disrupt the vacuolar membrane and replicate freely in the cytosol, *Shigella* and *Listeria.* Mutants which are unable to disrupt the vacuolar membrane do not replicate, but are not killed.

Fully fusogenic

Finally, a limited group of pathogens resides permanently in acidified vacuoles which are actively and rather nonselectively fusogenic with compartments of the endocytic cascade. These include *Coxiella burnetii* and *Leishmania spp.* Mature leishmania vacuoles intersect with fluid phase endocytic tracers, and are also enriched for LAMPs and other markers of late endosomes. Coxiella vacuoles fuse ubiquitously with terminal lysosomes, and efficiently with large vacuoles containing other pathogens or inert particles. Since these latter vacuoles ordinarily do not undergo efficient homotypic fusion, coxiella vacuoles function as "fusion machines," presumably as a consequence of efficient recruitment and retention of the fusion machinery for multiple steps in the endocytic cascade. Both leishmania and coxiella are dependent upon vacuole acidification for pathogen replication, and exaggerated vesicular traffic into and out of the vacuole is the most likely mechanism for nutrient acquisition.

Conclusions

Membrane traffic in pathogen-infected cells defies simple categorization. Subtle or obvious differences when well-studied pathogens are compared cannot necessarily be ascribed to avoidance of intracellular killing by lysosomal enzymes or acidic pH. They are likely in many cases to represent specialized mechanisms for nutrient acquisition. Defining these mechanisms will likely provide new strategies for therapeutic intervention.

Acknowledgments

The author thanks Norma Andrews, Stan Falkow, Brett Finlay, and Anthony Sinai for critical comments on the manuscript.

Preparation of the manuscript was supported by grants from the National Institutes of Health (AI-30060 and AI-31808) and by a Burroughs Wellcome fund Scholar Award in Molecular Parasitology.

References

1. Portillo, G.-d., and B.B. Finlay. 1995. The varied life-styles of intracellular pathogens within eukaryotic compartments. *Trends Microbiol.* 3:373–380.

2. Sinai, A.P., and K.A. Joiner. 1997. Safe haven: the cell biology of nonfusogenic pathogen vacuoles. *Ann. Rev. Microbiol.* In press.

3. Rothman, J.E. 1994. Mechanisms of intracellular protein transport. *Nature (Lond.).* 372:55–63.

4. Ikonen, E., M. Tagaya, O. Ullrich, C. Montecucco, and K. Simons. 1995. Different requirements for NSF, SNAP, and rab proteins in apical and basolateral transport in MDCK cells. *Cell.* 81:571–580.

5. Alvarez-Dominguez, C., A.M. Barbieri, W. Beron, A. Wandinger-Ness, and P.D. Stahl. 1996. Phagocytosed live *Listeria monocytogenes* influences Rab5 regulated in vitro phagosome endosome fusion. *J. Biol. Chem.* 271:13834–13843.

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