Current Biology, Vol. 15, 360–365, February 22, 2005, ©2005 Elsevier Ltd All rights reserved. DOI 10.1016/j.cub.2005.01.049

Endocytic Delivery to Lysosomes Mediated by Concurrent Fusion and Kissing Events in Living Cells

Nicholas A. Bright, Matthew J. Gratian, and J. Paul Luzio*
Cambridge Institute for Medical Research and
Department of Clinical Biochemistry
University of Cambridge
Addenbrooke's Hospital
Hills Road
Cambridge, CB2 2XY
United Kingdom

Summary

In mammalian cells, macromolecules internalized by endocytosis are transported via endosomes for digestion by lysosomal acid hydrolases [1–3]. The mechanism by which endosomes and lysosomes exchange content remains equivocal [2–9]. However, lysosomes are reusable organelles because they remain accessible to endocytic enzyme replacement therapies [10] and undergo content mixing with late endosomes [5, 6]. The maturation model, which proposes that endosomes mature into lysosomes [9], cannot explain these observations. Three mechanisms for content mixing have been proposed. The first is vesicular transport, best supported by a yeast cell-free assay [11]. The second suggests that endosomes and lysosomes engage in repeated transient fusions termed "kiss-and-run" [4]. The third is that endosomes and lysosomes fuse completely, yielding hybrid compartments from which lysosomes reform [3, 5, 6, 7, 8], termed "fusion-fission" [2]. We utilized time-lapse confocal microscopy to test these hypotheses in living cells. Lysosomes were loaded with rhodamine dextran by pulse-chase, and subsequently late endosomes were loaded with Oregon green 488 dextran. Direct fusions were observed between endosomes and lysosomes, and one such event was captured by correlative electron microscopy. Fluorescence intensity analyses of endosomes that encountered lysosomes revealed a gradual accumulation of lysosomal content. Our data are compatible with a requirement for direct contact between organelles before content is exchanged.

Results and Discussion

Because the "vesicular transport," "kiss-and-run," and "fusion-fission" hypotheses of content mixing between late endosomes and lysosomes are based on observations of fixed cells and/or cell-free in vitro content-mixing assays, we decided to evaluate content mixing with different fluorochrome-conjugated dextrans in living cells. We established a live cell assay based on previous experiments where we showed by EM that when BSA gold conjugates were endocytosed by fluid phase uptake in normal rat kidney (NRK) fibroblastic cells >85% accumulated in electron-dense, mannose 6-phosphate receptor (MPR)-negative lysosomes with the remainder being in endosome-lysosome hybrids [6]. In NRK cells lysosomes, electron lucent late endosomes and hybrid organelles are heterogeneous in shape and size (0.3–4 μm diameter) with late endosomes and hybrid organelles having the ultrastructural appearance of multivesicular bodies.

In the present study, NRK cells were incubated for 4 hr in culture medium containing dextran conjugated to rhodamine followed by a 20 hr chase in conjugate-free culture medium. Endocytosis of the fluorochrome-conjugated marker and its subsequent chase through the endocytic compartments resulted in its accumulation in lysosomes. By immunofluorescence, these organelles were lgp120 positive, and by immunogold electron microscopy, they were electron dense (Figure S1). The cells were then incubated in medium containing dextran conjugated to Oregon green 488 for 10 min followed by a 5 min chase in conjugate-free medium. We have shown previously that in NRK cells, this period of uptake resulted in delivery of the endocytosed fluorochrome into late endosomes marked by MPR (Figure S2) but largely devoid of lysosomal acid hydrolases [6]. The cells were transferred to the heated stage of an inverted microscope equipped with a confocal laser scanning attachment. Time-lapse images were then recorded to determine how content mixing of the two fluorochrome-laden organelle populations was achieved.

Figure 1A (Movie 1) demonstrates that content mixing between an Oregon green 488-labeled endosome and rhodamine-labeled lysosome resulted as a consequence of a direct fusion event occurring within a single 30 s timeframe. After imaging for 750 s, the Oregon green 488-laden endosomal compartment (green; arrow) received a dramatic influx of rhodamine-labeled lysosomal content resulting in colocalization of the two fluorochromes. This sudden influx of lysosomal content was clearly evident if the time-lapse sequence of the red lysosome channel was viewed in false color to display fluorescence intensity (Figure 1A). At the beginning of the time course, the endosome indicated by the arrow contained no lysosomal content but between 750 and 780 s received a rapid influx of lysosomal content via a short connecting tubule (arrowhead). By observing the fluorescence intensity of lysosome-derived content in false color, we were also able to determine that between onset of imaging and the sudden fusion event, a low level of red fluorescence intensity derived from lysosomes had accumulated in the endosome. This observation was confirmed by a plot of the fluorescence intensity profile against time depicted by Region Of Interest (ROI) 1 (Figure 1B) and correlated with a decrease in the green:red fluorescence ratio (not shown) indicating a gradual rise in red fluorescence relative to green prior to the fusion event. This gradual rise in red fluorescence was a result of transient "kissing" events. By applying this analysis to other organelles, we were able to show that

*Correspondence: jpl10@cam.ac.uk

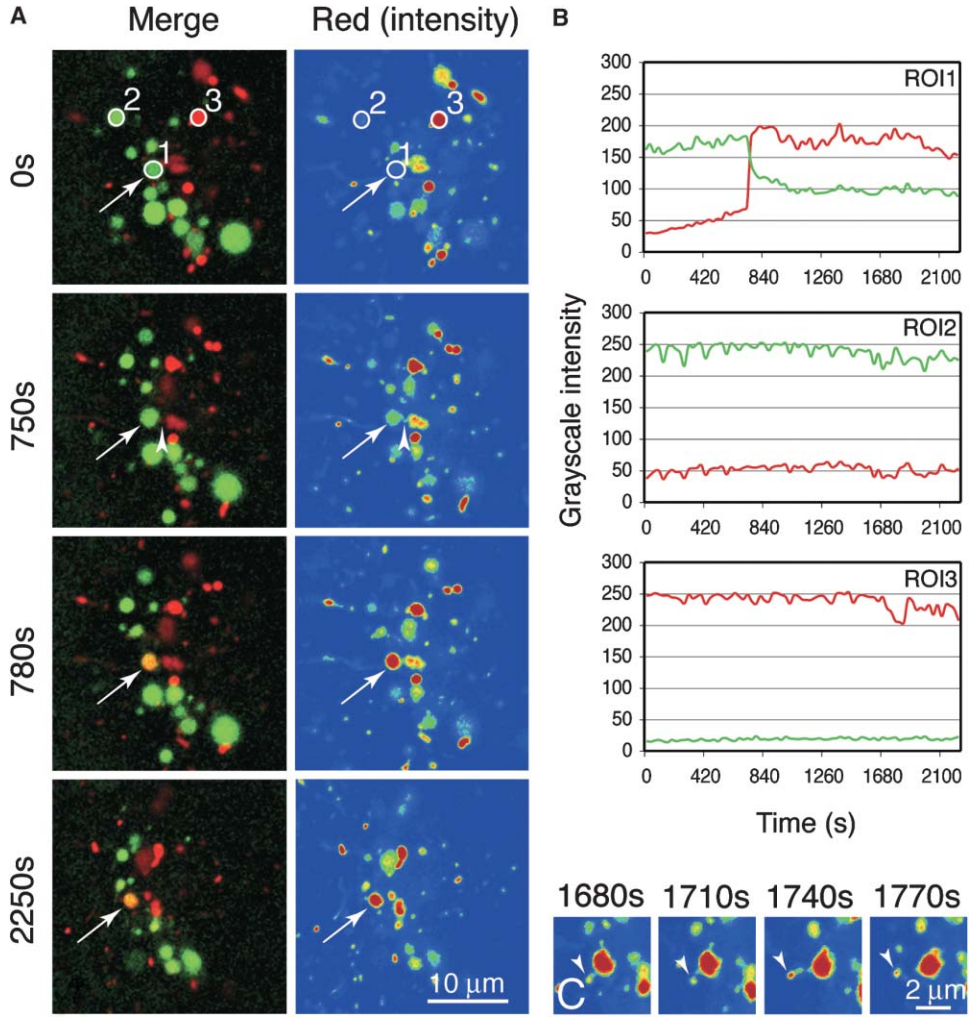

Figure 1. Endosome-Lysosome Fusion in a Living Cell

Images were recorded at 30 s intervals to evaluate content mixing between endosomes (green) and lysosomes (red). In Movie 1, they are played back at 10 frames/s. (A) A direct fusion (arrow) via a short connecting tubule (arrowhead) was observed after 780 s, resulting in a hybrid compartment (yellow). False color images showing red fluorescence intensity reveal accumulation in the endosome (arrow) prior to fusion. Low fluorescence intensity is depicted in blue, intermediate intensities are depicted in green and yellow, and high intensity fluorescence is depicted in red. (B) Fluorescence intensity of Oregon green (green line) and rhodamine (red line) in organelles denoted by ROI1, ROI2, and ROI3. The fluorescence intensity of organelles that did not interact remained unchanged. (C) After the fusion (ROI1), budding profiles were observed pinching off the hybrid organelle (arrowheads).

Oregon green 488-laden endosomes (ROI2; Figure 1B) and rhodamine-laden lysosomes (ROI3; Figure 1B), which did not undergo interactions throughout the time course, did not undergo a change in fluorescence intensity.

We have previously proposed that lysosomes fuse with late endosomes to generate hybrid compartments in which digestion occurs [3]. However, this would rapidly lead to a depletion of dense core lysosomes within the cell, and, thus, we have speculated that lysosomes are reformed from hybrid compartments by maturation events including retrieval of membrane, export of digested products, and recondensation of lysosome content. After the fusion event occurring by 780 s in the sequence shown in Figure 1A, there was a lag followed by the emergence of budding profiles from the hybrid compartment depicted by ROI1 (Figure 1). These buds, which had formed by 1680 s, subsequently detached (Figure 1C) consistent with membrane retrieval and maturation events occurring during dense core lysosome reformation.

Some endosomes that engaged lysosomes in repeated interactions gradually accumulated lysosome-derived dextran-rhodamine content in the absence of apparent fusion events (Figure 2A and Movie 2). A plot of the fluorescence intensity of the identified endosome (ROI1) against time (Figure 2B) demonstrated a steady accumulation of red fluorescence derived from the neighboring lysosomes that frequently docked onto the endosome periphery throughout the movie sequence. These observations provided evidence for repeated transient interactions between endosomes and lysosomes whereby organelle identity was retained after exchange of content as predicted by the "kiss-and-run"

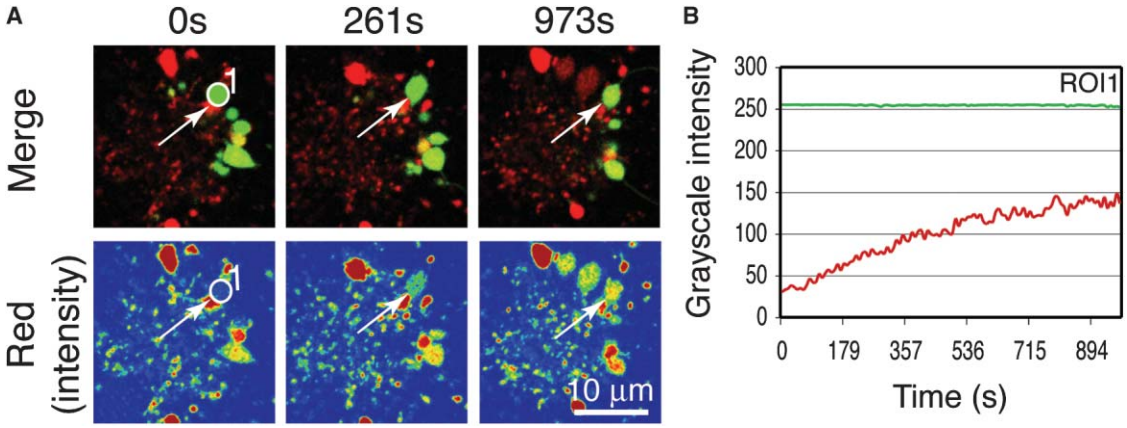

Figure 2. "Kiss-and-Run" Content Mixing in a Living Cell

Images were recorded at 9 s intervals. In Movie 2, they are played back at 10 frames/s. (A) Gradual accumulation of lysosomal content (red) was observed in an endosome (green). False color images showing red fluorescence intensity reveal an accumulation of red fluorescence in the endosome indicated (arrow). (B) Fluorescence intensity of Oregon green (green line) and rhodamine (red line) in the endosome denoted by ROI1. At the onset of imaging this endosome was devoid of red fluorescence, but there was a gradual accumulation throughout the sequence indicating transient "kiss-and-run" delivery of red fluorescence independent of direct fusion.

model [4]. Endosomes and lysosomes that did not engage in direct physical contact did not exchange content indicating that small transport vesicles, beyond the resolution of the light microscope (i.e., <0.2 μm), were not responsible for content mixing. From an analysis of forty independent content-mixing events in living cells, 32% were direct fusions, the remainder being "kiss-and-run" or "kiss-and-linger" [12] events. The size of the organelles had no influence on which of the mechanisms was used. Content mixing by "kissing" often preceded fusion but was not a prerequisite for it (see Figure S3, which shows an endosome fusing sequentially with two separate lysosomes without observable kissing events). We do not know whether endosomes that have received lysosomal content from kissing events are more susceptible to fusion, only that fusion was not observed in the majority of cases where content mixing occurred during the time courses of our live cell imaging.

The data presented in Figure 1 indicated the presence of an interconnecting tubule between a lysosome and an adjacent endosome with which the lysosome subsequently fused. Tubulation of endocytic compartments in live cells has been demonstrated previously [13–15]. We observed other events in which content mixing between organelles was mediated through a connecting tubule, often over long distances. Figure 3A (Movie 3A) shows an endosome-lysosome hybrid (green with some red content) from which tubules could be seen to project, and at the end of the sequence, one of these attached to a nearby lysosome and mediated content mixing before the two compartments fused completely. An analysis of the fluorescence intensity of the lysosome (ROI1, Figure 3B) clearly demonstrated an influx of Oregon green 488 before the organelles fused. Fusion between late endosomes and lysosomes was also mediated via tubules derived from lysosomes. Figure 3C (Movie 3C) demonstrates a rhodamine-laden lysosome that exchanged content with an Oregon green 488-laden late endosome via a tubule derived from the lysosomal compartment. This tubule extended 13.5 μm from the lysosome and contained only lysosomal content at the start of the sequence. The tubule fused with a distant endosome mediating bidirectional exchange of lumenal content. It is clear in sequences from which the red (lysosomal) channel has been removed that content from the endosome flooded through the connecting tubule into the lumen of the lysosome from which it was derived (Figure 3C). An analysis of fluorescence intensities of these organelles supports these observations (Figure 3D). Tubulation from either organelle population was abolished by treatment with nocodazole indicating microtubule dependence (data not shown) in agreement with a role for microtubules in tubulation dependent fusion of phagosomes [16].

To determine the ultrastructure of compartments that exchanged content, we used correlative live cell and electron microscopy (CLEM) [17]. By definition, we have usually added fixative to the live cells before or after a fusion event. However, in one instance, we captured the moment of direct fusion. Figure 4A (and Movie 4) shows after imaging for 712 s a lysosome appear in the confocal plane and attach to a hybrid compartment (ROI1). A fluorescence intensity profile of ROI1 (data not shown) indicated a gradual rise in red fluorescence during the subsequent time course. Fixative was applied immediately after the final frame (1007 s; Figure 4B) and a Differential Interference Contrast (DIC) image (Figure 4C), and merged fluorescence and DIC image (Figure 4D) were recorded to enable subsequent identification of the organelles in the electron microscope. After processing for transmission EM, we were able to identify the structures that we had imaged in the living cell (Figure 4E, arrows). These hybrid compartments contained numerous internal membranes, vesicles, and diffuse electron dense content. We were also able to identify the lysosome (Figure 4F, arrowhead) that we had observed dock onto the surface of the hybrid structure after 756 s of imaging. Serial sections revealed that fixation had occurred as the lysosome fused with the hybrid organelle, as indicated by the release of lysosomal contents

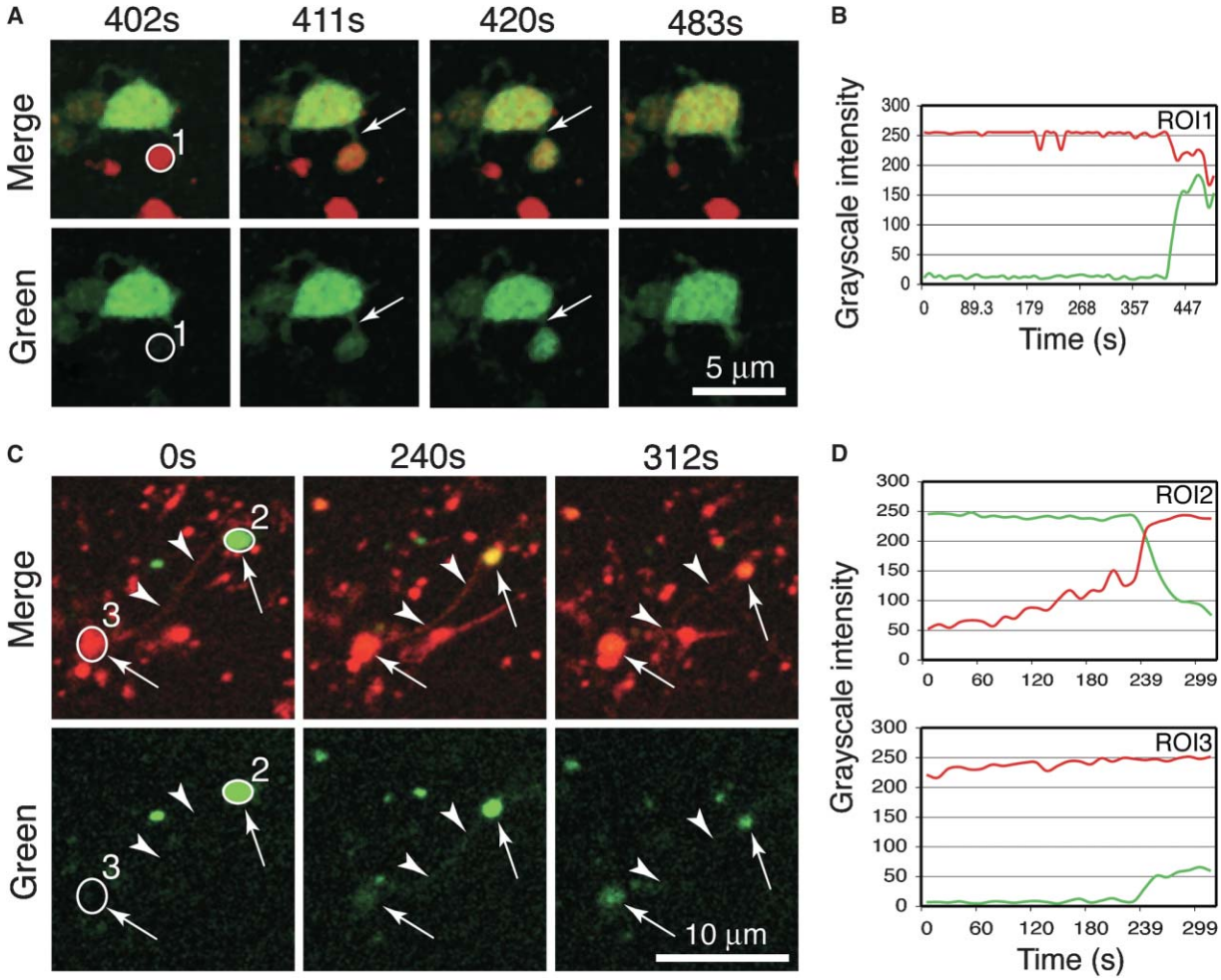

Figure 3. Fusion Mediated via Tubules

Images were recorded at 9 s intervals (a) and 12 s intervals (c). In Movies 3a and 3c, they are played back at 10 frames/s. (A) Content mixing between an endosome-lysosome hybrid (green with some red content) and a lysosome (red) was mediated via a tubule (arrow) derived from the hybrid. Subsequently the hybrid and lysosome fused completely. Corresponding images show the green channel only. (B) Fluorescence intensity of Oregon green (green line) and rhodamine (red line) in the lysosome denoted by ROI1. (C) A fusion between an endosome (green) and a lysosome (red; arrows) was mediated via a 13.5 μm tubule (arrowheads) derived from the lysosome. Corresponding images show the green channel only. (D) Fluorescence intensity of Oregon green and rhodamine in the endosome denoted by ROI2 and the lysosome denoted by ROI3.

into the hybrid lumen (Figure 4G). These data reveal that after an initial content-mixing event yields a hybrid intermediate compartment, the organelle remains accessible to multiple fusion (Figure S3) or kissing events as predicted from our cell-free content-mixing assay [5]. Upon direct fusion, a bolus of lysosomal content can clearly be released into the lumen of the fusing organelle where it is able to diffuse into the aqueous environment.

In summary, the content mixing observed in our experiments had the following characteristics: (1) it was only seen between organelles in physical contact, (2) no vesicular traffic delivering content between endosomes and lysosomes was observed, (3) both kissing and direct fusion events were seen, with kissing sometimes, but not always, preceding fusion, (4) tubulation of the organelles sometimes occurred and tubules were able to exchange content with organelles by both kissing and direct fusion events. It has been recognized that "kiss-and-run" and "fusion-fission" hypotheses are not mutually exclusive [2, 3]. However, neither readily accounts for the behavior of lysosomes in full. "Kiss-and-run" transient fusion events cannot easily be used to explain the generation of hybrid compartments, intermediate in density between late endosomes and lysosomes in cell-free in vitro content-mixing assays [5]. Nor may it easily account for observations that flocculant aggregates of endocytosed BSA gold, up to a micron or more in diameter when preloaded into lysosomes, are capable of being delivered from lysosomes to hybrid intermediate compartments [6]. Conversely, the "fusion-fission" hypothesis involving direct fusion cannot easily account for the observation that late endocytic compartments act as molecular sieves and separate different sizes of fluorochrome-conjugated dextran molecules [18, 19], implying discrete exchange at a molecular level.

Our data help to resolve the conflict between the "kiss-

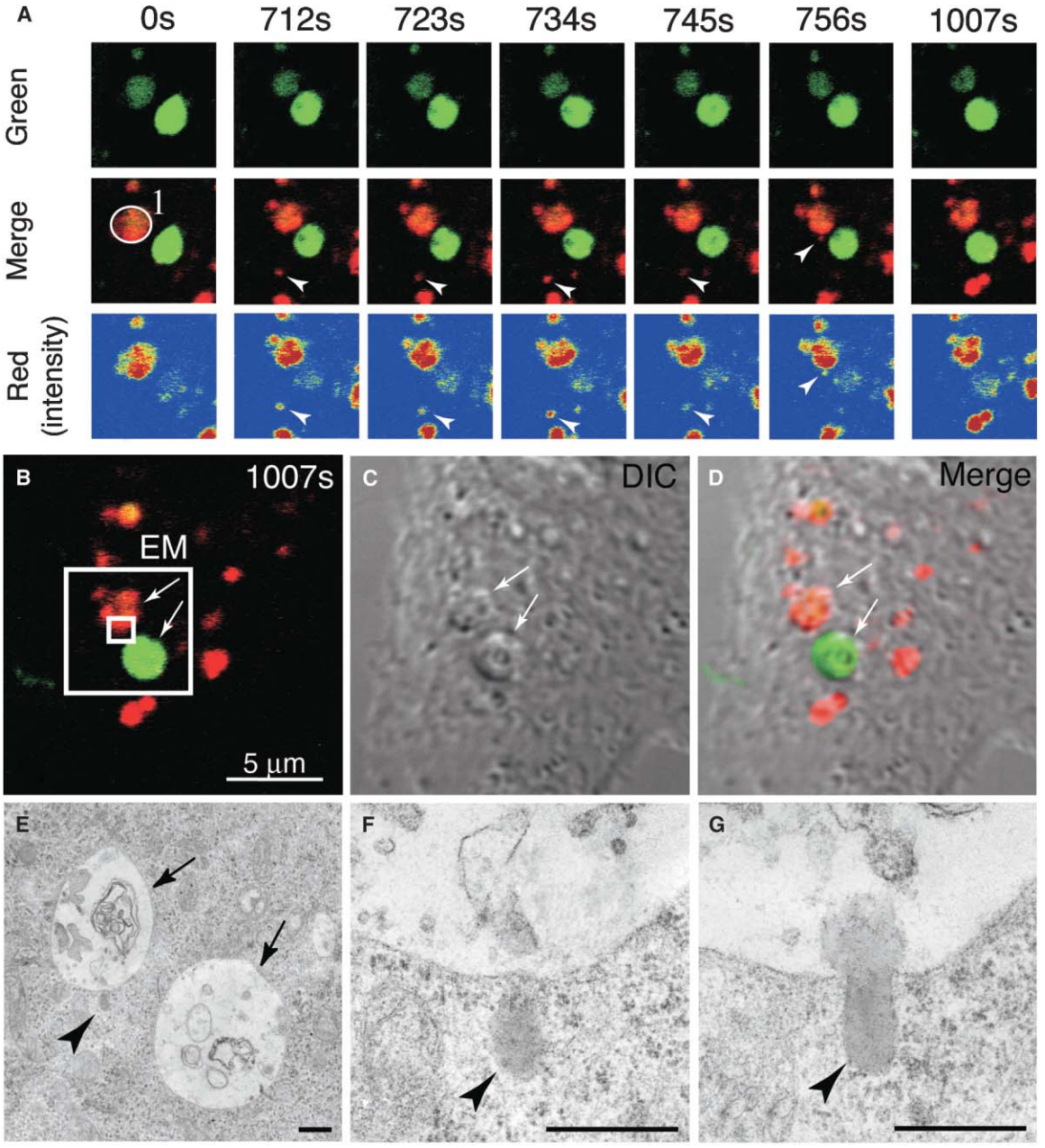

Figure 4. CLEM of a Fusion

Images were recorded at 11 s intervals. In Movie 4, they are played back at 10 frames/s. (A) A lysosome (arrowhead) appeared in the confocal plane after 712 s and docked onto a hybrid organelle (ROI1) after 756 s. Red (intensity) false color images indicate the fluorescence intensity of the red fluorochrome at various time points. The last time point (B), a DIC micrograph (C), and merged overlay (D) enabled identification of these organelles (arrows) by EM (E). Arrowheads (E–F) show the lysosome that docked onto the hybrid organelle in ROI1. A higher magnification of this lysosome is shown (F). Serial sections revealed that the cell had been fixed at the moment the organelles fused (G). EM scale bars, 500 nm.

and-run" and "fusion-fission" hypotheses by demonstrating that direct fusion and transient exchange via kissing occur concurrently in living cells. In our experiments, these mechanisms account for all content transfer between late endosomes and lysosomes. The use of CLEM has provided the observation of a fusion event at the ultrastructural level. In future experiments using antibodies to immunolabel components of the fusion machinery [7, 8, 20, 21], CLEM will afford us the opportunity of mapping molecular machinery onto the "kiss-and-run" and "fusion-fission" mechanisms outlined.

Supplemental Data

Supplemental Data including Experimental Procedures are available with this article online at http://www.current-biology.com/cgi/content/full/15/4/360/DC1/.

Acknowledgments

This work was funded by a Programme Grant (G9310915) from the Medical Research Council to J.P.L. Cambridge Institute for Medical Research is supported by a Strategic Initiative Grant (066438) from the Wellcome Trust. The Zeiss LSM510 microscope was purchased with an equipment grant (062658) from the Wellcome Trust. We thank our colleagues Margaret Robinson, David Owen, and Folma Buss for helpful discussions and comments on our manuscript.

Received: October 19, 2004
Revised: December 14, 2004
Accepted: December 16, 2004
Published: February 22, 2005

References

1. Gruenberg, J. (2001). The endocytic pathway: a mosaic of domains. Nat. Rev. Mol. Cell Biol. *2*, 721–730.
2. Mullins, C., and Bonifacino, J.S. (2001). The molecular machinery of lysosome biogenesis. Bioessays *23*, 333–343.
3. Luzio, J.P., Poupon, V., Lindsay, M.R., Mullock, B.M., Piper, R.C., and Pryor, P.R. (2003). Membrane dynamics and the biogenesis of lysosomes. Mol. Membr. Biol. *20*, 141–154.
4. Storrie, B., and Desjardins, M. (1996). The biogenesis of lysosomes: is it a kiss and run, continuous fusion and fission process? Bioessays *18*, 895–903.
5. Mullock, B.M., Bright, N.A., Fearon, C.W., Gray, S.R., and Luzio, J.P. (1998). Fusion of lysosomes with late endosomes produces a hybrid organelle of intermediate density and is NSF dependent. J. Cell Biol. *140*, 591–601.
6. Bright, N.A., Reaves, B.J., Mullock, B.M., and Luzio, J.P. (1997). Dense core lysosomes can fuse with late endosomes and are reformed from the resultant hybrid organelles. J. Cell Sci. *110*, 2027–2040.
7. Pryor, P.R., Mullock, B.M., Bright, N.A., Gray, S.R., and Luzio, J.P. (2000). The role of intraorganellar Ca^{2+} in late endosome-lysosome heterotypic fusion and in the reformation of lysosomes from hybrid organelles. J. Cell Biol. *149*, 1053–1062.
8. Pryor, P.R., Mullock, B.M., Bright, N.A., Lindsay, M.R., Gray, S.R., Richardson, S.C., Stewart, A., James, D.E., Piper, R.C., and Luzio, J.P. (2004). Combinatorial SNARE complexes with VAMP7 or VAMP8 define different late endocytic fusion events. EMBO Rep. *5*, 590–595.
9. Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. (2002). Molecular Biology of the Cell, Fourth Edition, (New York: Garland Science).
10. Desnick, R.J., and Schuchman, E.H. (2002). Enzyme replacement and enhancement therapies: lessons from lysosomal disorders. Nat. Rev. Genet. *3*, 954–966.
11. Vida, T., and Gerhardt, B. (1999). A cell-free assay allows reconstitution of vps33p-dependent transport to the yeast vacuole/lysosome. J. Cell Biol. *146*, 85–97.
12. Bezzerides, V.J., Ramsey, I.S., Kotecha, S., Greka, A., and Clapham, D.E. (2004). Rapid vesicular translocation and insertion of TRP channels. Nat. Cell Biol. *6*, 709–720.
13. Hopkins, C.R., Gibson, A., Shipman, M., and Miller, K. (1990). Movement of internalised ligand-receptor complexes along a continuous endosomal reticulum. Nature *346*, 335–339.
14. Boes, M., Cerny, J., Massol, R., Op den Brouw, M., Kirchhausen, T., Chen, J., and Ploegh, H.L. (2002). T-cell engagement of dendritic cells rapidly rearranges MHC class II transport. Nature *418*, 983–988.
15. Chow, A., Toomre, D., Garrett, W., and Mellman, I. (2002). Dendritic cell maturation triggers retrograde MHC class II transport from lysosomes to the plasma membrane. Nature *418*, 988–994.
16. Harrison, R.E., Bucci, C., Vieira, O.V., Schroer, T.A., and Grinstein, S. (2003). Phagosomes fuse with late endosomes and/or lysosomes by extension of membrane protrusions along microtubules: role of Rab7 and RILP. Mol. Cell. Biol. *23*, 6494–6506.
17. Koster, A.J., and Klumperman, J. (2003). Electron microscopy in cell biology: integrating structure and function. Nat. Rev. Mol. Cell Biol. Suppl. *4*, SS6–SS10.
18. Wang, Y.-L., and Goren, M.B. (1987). Differential and sequential delivery of fluorescent lysosomal probes into phagosomes in mouse peritoneal macrophages. J. Cell Biol. *104*, 1749–1754.
19. Berthiaume, E.P., Medina, C., and Swanson, J.A. (1995). Molecular size-fractionation during endocytosis in macrophages. J. Cell Biol. *129*, 989–998.
20. Antonin, W., Holroyd, C., Fasshauer, D., Pabst, S., Von Mollard, G.F., and Jahn, R. (2000). A SNARE complex mediating fusion of late endosomes defines conserved properties of SNARE structure and function. EMBO J. *19*, 6453–6464.
21. Antonin, W., Fasshauer, D., Becker, S., Jahn, R., and Schneider, T.R. (2002). Crystal structure of the endosomal SNARE complex reveals common structural principals of all SNAREs. Nat. Struct. Biol. *9*, 107–111.