Arguments in favour of endosome maturation

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Endocytosed substances enter the endocytic pathway via early endosomes, which segregate proteins that recycle to the plasma membrane from proteins that are ultimately targeted to lysosomes. Before reaching lysosomes, endocytosed proteins can be detected first in late endosomes, at which the majority of newly synthesized lysosomal proteins enter the endocytic pathway. Early and late endosomes are often differentially localized in the cell; early endosomes are usually found at the periphery of the cell, whereas late endosomes are more frequently detected near the microtubule-organizing centre. These localizations are, however, not absolute, and depend strongly on the cell type that is studied. Endosomes are pleomorphic. Although both typical early and typical late endosomes can be found in most cell types, many endosomes have intermediate morphological features. Vacuolar and tubular membraneous structures containing endocytic markers can be distinguished both for typical early and for typical late endosomes. Depending on the cell type, endosomal tubules vary in length up to an extensive reticulum in some cell types [1–3]. The vacuolar late endosome is usually larger than the vacuolar early endosome, and late endosomal vacuoles contain many internal vesicles (also referred to as multivesicular bodies). However, many vacuolar endosomes are intermediate in size and may contain just a few intravacuolar vesicles.

The mechanism(s) by which ligands are transported from early to late endosomes and finally to lysosomes is still a matter of debate. At present, at least two models have been proposed [4], and many of the arguments supporting each model have been reviewed elsewhere [5, 6].

Models for transport from early to late endosomes

The first model predicts that early and late endosomes are pre-existing compartments that communicate via vesicular traffic, a process that is independent of the vesicular traffic between the plasma membrane and the early endosomes. In baby hamster kidney cells it has been described that horseradish peroxidase (HRP), when endocytosed as a fluid-phase marker, was detectable in early endosomes after 2 min of uptake, whereas HRP was detected in late endosomes only after 15 min of uptake [7, 8], indicating a sequential arrival in the two compartments. When early and late endosomes are pre-existing organelles with a fixed localization in the cell, carrier vesicles may actively transport endocytosed material from early to late endosomes. Indeed, putative carrier vesicles have been described, linked to microtubules by means of CLIP170 [9], which may be transported to the minus end of microtubules using dynein as a motor protein. However, CLIP170 has also been shown to co-localize with a typical early endosomal marker, transferrin, indicating that early endosomes may be linked to microtubules as well. The recovery of endocytosed ligands from early, but not from late endosomes, after incubation at 18°C [10, 11], or in

Abbreviations used: ASGP, asialoglycoprotein; ASGPR, asialoglycoprotein receptor; HRP, horseradish peroxidase; LDL, low-density lipoprotein; MPR, mannose-6-phosphate receptor; Tf, transferrin; TIR, transferrin receptor; TGN, trans-Golgi network.
the presence of the microtubule-depolymerizing drug, nocodazole [12] supports the notion of sequential passage of endocytosed proteins through early and late endosomes respectively. The accumulation of endocytic marker-containing spherical vesicles, detected in the presence of the nocodazole, that were distinct from early endosomes and from acid-phosphatase-positive lysosomes, suggested that they may be responsible for transport from early to late endosomes [12].

The second model for transport through the endosomal system involves the continuous fusion and fission of vesicles that derive from or are destined for the plasma membrane with endosomes. During this process, early endosomes gradually mature into late endosomes. The major difference between the two models is that, in the stable compartment model, at least two types of transport vesicles that bud off from the early endosome must be generated: one which pinches off from the endosomal tubules, transport receptors and ligands that remain receptor-associated to the plasma membrane, and another class, carrier vesicles, that fuse with late endosomes. In contrast, the maturation model (see below) requires only one class of vesicles to bud from the endosome to mediate transport to the plasma membrane. As mentioned above, putative carrier vesicles have been described in nocodazole-treated cells. However, it has also been documented that vesicular transport from the trans-Golgi network (TGN) to endosomes is inhibited in the presence of nocodazole (reviewed in [13]). Since this is the major pathway by which newly synthesized lysosomal enzymes and their carrier, the mannose-6-phosphate receptor (MPR), are targeted to the degradative pathway, the entry of these proteins into the endocytic pathway is inhibited. Therefore, the perturbed transport from the TGN to endosomes at reduced temperatures and in the presence of nocodazole may lead to the incomplete maturation of endosomes and the depletion of MPR in artificial endosomal subtypes that are formed at these conditions. However, whether the observed accumulation of ‘carrier vesicles’ is a result of the inhibition of transport to late endosomes, or of inhibition of the formation of late endosomes remains to be established. Maturation of endosomes under physiological conditions may occur in parallel with their translocation to the microtubule-organizing centre, thus explaining their differential localization.

As well as topological and morphological differences, there are also biochemical differences between early and late endosomes. In pre-existing early and late endosomes, the exclusive presence of certain proteins, defining the compartments, could be expected. However, differences in the protein composition of early and late endosomes may also be due to the enrichment of passenger proteins, rather than the presence of resident proteins. The overall protein content of early and of late endosomes is very similar [14]. Differences in the relative amount of some specific proteins have been detected, but, to date, no proteins have been found that are exclusively present either in early or in late endosomes. Endocytosing receptors, such as the transferrin (Tf) receptor, the MPR and lysosomal glycoproteins have been reported to be specific markers of early endosomes, late endosomes and lysosomes respectively. However, we and others have reported that these markers can be detected in the entire endocytic track [15–17]. Two distinct GTP-binding proteins, rab5 and rab7, are strongly enriched in early and in the late endosomes respectively [18–20]. The maturation model would argue for a more gradual transit of rab5-positive to rab7-positive endosomes. However, for proteins that are transiently attached to the cytoplasmic side of the endosomes, such as the rab proteins, a rather sudden change in affinity may occur when the binding is dependent on threshold values of intrinsic properties of the endosomes, such as the luminal pH and the membrane potential. It has been reported that the luminal pH of early and of late endosomes is different, and that endocytosed ligands encounter pH values ranging gradually from 6.3–5.0 [21, 22]. A gradual decrease of endosomal pH is exactly what would be expected when early endosomes mature into late endosomes.

**Endosomal-maturation model**

My colleagues and I have used asialoglycoproteins (ASGPs) and Tf as markers of the degradative-endocytic pathway and of the receptor-recycling pathway, respectively. Both the ASGP receptor (ASGPR) and the Tf receptor (TfR), and their ligands, enter the cell via coated vesicles [1, 23, 24] with a half-time of 2–3 min [25, 26]. ASGP dissociates from its receptor in endosomes, and is subsequently transported to lysosomes. Tf remains associated with its receptor until the entire complex has recycled to the plasma membrane. The presence of the TfR and of ASGP within the same intracellular vesicles has been described in several studies [27, 28], including our own, in which we showed that ASGP is efficiently sorted from Tf immediately after receptor-mediated uptake [29], and that the TfR and the ASGPR follow exactly the
same endocytic pathway [30]. Although all endocytosed Tf must pass through sorting endosomes, only a little was found within these compartments [17], indicating that the passage of Tf through the sorting endosomes is extremely rapid. Sorting between simultaneously endocytosed Tf and ASGP also occurred extremely rapidly [29]. Since the half-time for the recycling of the TIR in hepatoma (HepG2) cells at 37°C is 5 min [26], we concluded that the majority of intracellular Tf localizes within transport vesicles/tubules between the endosomes and the plasma membrane, rather than in sorting endosomes.

We proposed a model of endosome maturation in which vesicular transport between the plasma membrane and individual endosomes gradually diminishes in time, but continues all the way up to late endosomes [17, 30]. This model is based on a number of findings, as follows. (1) Percoll gradients have previously been shown to separate endosomes from lysosomes on the basis of their buoyant densities (for a review see [31]). We used shallow-Percoll-density gradients to separate early from late endosomes, and demonstrated that endocytosed ASGP was retrieved from vesicles that gradually increased in buoyant density at increasing times from uptake [17, 29]. (2) The endocytic pathway that was travelled by a pulse of endocytosed ASGP remained accessible to a short pulse of sequentially endocytosed Tf for 20 min after uptake. Early and late endosomes were accessed by endocytosed Tf with identical kinetics, indicating that some endocytosed Tf (and hence some of the TIR) is bypassing early endosomes and is transported directly to late endosomes [17]. (3) Within the endocytic pathway, the bulk of MPR is found in late endosomes [10, 15], whereas TIRs are recovered mainly in early endosomes. About 10% of MPR is expressed at the plasma membrane and is in equilibrium with intracellular MPR. Sorting of endocytosed MPR from TIR-containing compartments occurs at about 20 min after uptake [30]. However, a maximal amount of 50% of the intracellular cation-dependent MPR was accessible to endocytosed Tf within 5 min, with kinetics that were identical to those for the TIR. This finding is consistent with direct transport of endocytosed Tf both to early and to late endosomes, rather than transport of Tf via early to late endosomes. Only the maturation model described above can explain the apparent discrepancy between the late sorting of endocytosed MPR from recycling TIRs, and the much more rapid access of intracellular MPR to the TIR. (4) After continuous uptake of Tf for 5 min, we were able to confirm the presence of Tf in at least 85% of MPR containing endosomes using immunoelectron microscopy [17]. Moreover, at least 82% of the endocytosed Tf was localized in MPR-containing endosomes. We found no indication of separate pools of endosomes by quantitating labelling for MPR and Tf in individual endosomes, that is, early endosomes that were rich in Tf/HRP and poor in MPR, and late endosomes poor in Tf/HRP but rich in MPR. Instead, the results were compatible with a gradual maturation of early into late endosomes.

Since the distribution of newly formed endocytic structures on Percoll-density gradients was different from that of 15-min-old endosomes, we concluded that the majority of ligand was targeted to early endosomes, and that the accessibility of endosomes to transport vesicles decreased over time [17]. However, direct transport of Tf/HRP to late endosomes was still considerable for the following reasons. (1) Fusion of a single endocytic vesicle with a late endosome results in the transfer of many Tf molecules to this compartment. (2) If the percentage of late endosomes fusing with endocytic vesicles is determined by a Poisson distribution, on average many Tf-containing endocytic vesicles must have fused with each single late endosome to access nearly all late endosomes. (3) Although the distinction between early and late endosomes becomes arbitrary in an endosomal maturation model, the morphological data show that significant amounts of Tf are being transported to MPR-rich late endosomes.

The endosome-maturation model is consistent with in vitro studies, which also demonstrate a decrease in the fusogenicity of endosomes that are isolated at later times after ligand uptake [31–34]. Hopkins and colleagues [35] have proposed a model in which endocytosed markers enter a network of tubular cisternae with discrete swellings that move along the tubes. These swellings are analogous to the vesicular-endosomal compartment, and may be the sites of unbound-ligand accumulation. We and others have also detected long tubular extensions of endosomes in various cell types, and have proposed connections between early and late endosomes via tubular extensions [1, 3, 15, 23, 36, 37]. In the presence of brefeldin A, the length of endosomal tubules and the number of endosomal connections significantly increased (W. Stoorvogel, unpublished results) [38–41], indicating that the experimental conditions used did not give rise to artificial short endosomal tubules. It can therefore be concluded that endosomes within one cell do not form a single continuous network,
although in certain cell types endosomal tubules may reach a considerable length. However, by forming temporal tubular connections, endosomes may be continuous in time.

Salzman and Maxfield [11] have shown that, in a Chinese-hamster ovary derived cell line, vacuoles in the endocytic pathway that are labelled with a short pulse of endocytosed $a_{1}$-macroglobulin became inaccessible for a second pulse of ligand with a half-time of about 8 min. They argued that the half-time may reflect the time required for a maturing endosome to become fusion-inaccessible for plasma-derived vesicles. In a second article [42], the same group reported three additional observations that argue in favour of endosome maturation. (1) While chasing a pulse of endocytosed low-density lipoprotein (LDL) into the cell, the ligand content per endosome was unchanged. (2) The number of labelled endosomes per cell did not change during the chase. (3) An increasing number of endosomes that were not apparent after a first pulse of LDL became labelled with a second pulse of LDL. In contrast with this group, we reported that a pulse of endocytosed ASGP in HepG2 cells that was chased into late endosomes was accessible to a second pulse of ligand, with kinetics identical to those for early endosomes [17], and concluded that late endosomes can fuse directly with plasma-membrane-derived endocytic vesicles. Although we agree with this group that transport through the endocytic pathway occurs by means of endosome maturation, we disagree on the fusion accessibility of late endosomes for plasma-membrane-derived vesicles. However, the data that form the basis of these different views are not necessarily opposed. Maxfield and coworkers have measured the percentage of endosomes that become fusion inaccessible in time, whereas we measured the percentage of molecules that were endocytosed in the first pulse that became inaccessible for a second pulse of endocytosed molecules. If, as we proposed, all the endosomes fuse with plasma-derived-endocytic vesicles and the majority of these vesicles fuse with relatively early endosomes, both early and late endosomes will be fluorescently labelled, although with a different intensity, after a first short pulse of ligand uptake. In this scenario the results of Maxfield and coworkers could be interpreted as reflecting maturation of late endosomes into lysosomes, rather than the maturation of early endosomes into late endosomes. In this respect, it would be interesting to know whether, after a pulse of fluorescent ligand, the fusion of subsequently formed endocytic vesicles with brightly labelled (early) endosomes will decrease with a half-time that is longer than the half-time for relatively weakly labelled (late) endosomes. If so, a strong argument for the maturation of late endosomes into lysosomes would have been made.

A proportion of the endocytosed TRRs that enter late endosomes may be transported sequentially to the TGN. Active vesicular shuttling between the late endosomes and the TGN is thought to govern the transport of newly synthesized hydrolases to lysosomes via MPR [10, 13, 15]. Consequently, some TRR could be expected to be transported to the TGN as well. This notion is strengthened by our previous findings that, in HepG2 cells, a proportion of endocytosed Tf enters the TGN, where it is mixed with newly synthesized secretory protein [43]. This process occurred in less than 10 min, which is consistent with the rapid entry of Tf into late endosomes.

Conclusions

In summary, we have demonstrated that, in time, endosomes gradually increase in buoyant density, until a buoyant density near to that of lysosomes is reached. During this process, endosomes remain accessible to recycling receptors, with unchanged kinetics. Furthermore, during maturation, endosomes decrease in TRR contents, but increase in MPR contents. These data are most consistent with a model of endosomal maturation. In such a model, plasma-membrane-derived receptor-containing vesicles fuse with all, but preferably with early, endosomes. Similarly, early endosome-derived vesicles fuse preferably with the plasma membrane. Endosomes mature during this fusion and fission process, during which they gradually decrease their interaction with the plasma membrane. Simultaneously, the intensity of membrane exchange with the TGN is increased. Receptors that function in receptor-mediated endocytosis, such as the TfR, preferentially exit from endosomes immediately after their arrival in them, and consequently shuttle mainly between the plasma membrane and early endosomes. MPRs, however, preferentially exit from late endosomes, and are thus transported to the TGN.


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