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Exosomes – vesicular carriers for intercellular communication

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Cells release different types of vesicular carriers of membrane and cytosolic components into the extracellular space. These vesicles are generated within the endosomal system or at the plasma membrane. Among the various kinds of secreted membrane vesicles, exosomes are vesicles with a diameter of 40–100 nm that are secreted upon fusion of multivesicular endosomes with the cell surface. Exosomes transfer not only membrane components but also nucleic acid between different cells, emphasizing their role in intercellular communication. This ability is likely to underlie the different physiological and pathological events, in which exosomes from different cell origins have been implicated. Only recently light have been shed on the subcellular compartments and mechanisms involved in their biogenesis and secretion opening new avenues to understand their functions.

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Introduction

Cells do not only use vesicular and tubulovesicular transport carriers to deliver cargo between membranes within a cell, but also generate vesicles that are secreted into the extracellular space. These extracellular membrane vesicles are thought to function as shuttles for the delivery of cargo between different cells within an organism. The vesicles that are found in the extracellular space of cells in tissue culture are diverse and depend on the origin of the cells and their current state (e.g. transformed, differentiated, stimulated, stressed). Extracellular vesicles such as exosomes, apoptotic blebs, microvesicles, microparticles, prostasomes and prominosomes have been named [1,2]. Some of these vesicles are poorly defined and it remains unclear to what extent they represent an overlapping spectrum of common classes of vesicles making it difficult to ascribe functions to each type [1].

Exosomes are the vesicles that have so far received most attention over the past recent years [3•,4,5]. Exosomes have been implicated in various different cellular functions and disease states where they could constitute valuable biomarkers [6,7]. Indeed most body fluids contain significant amounts of exosomes [8,9]. Nevertheless their physiological relevance has been difficult to evaluate because their origin, biogenesis and secretion mechanisms remained enigmatic. Here, we summarize our current view on exosome biogenesis and function and highlight studies that hopefully will bring light on their physiological relevance.

Defining exosomes

Although potentially any vesicle released by a cell carries cell type specific membrane and cytosolic components, there are a number of features that should be taken into consideration for the characterization of exosomes. Exosomes are between 40 and 100 nm in diameter, appear with a characteristic cup-shaped morphology (after negative staining) or as round well delimited vesicles as observed by transmission and cryo-electron microscopy, respectively [10]. Exosomes float on sucrose gradient to a density that ranges from 1.13 to 1.19 g/ml [11]. Apart from their morphology, their unique protein and lipid composition enable their identification. As a consequence of their endosomal origin, nearly all exosomes independently of the cell type from which they originate contain proteins involved in membrane transport and fusion (e.g. Rab GTPases, Annexins, flotillin), in multivesicular body (MVB) biogenesis (e.g. Alix and TSG101), in processes requiring heat shock proteins (hsc70 and 90), integrins and tetraspanins (e.g. CD63, CD9, CD81 and CD82). While some of the proteins that are found in the proteome of many exosomal membrane preparations may merely reflect the cellular abundance of the protein, others are specifically enriched in exosomes and can therefore be defined as exosomal marker proteins (e.g. Alix, flotillin, TSG101, CD63). Another feature of exosomes is their enrichment in raft-lipids such as cholesterol, sphingolipids, ceramide and glycerophospolipids with long and saturated fatty-acyl chains [12,13,14•].

Some cells, in particular tumour cells or for example platelets and monocytes, release often after stimulation (e.g. stress, activation, apoptosis) vesicles of larger size (100 nm–1 μm in diameter) and of heterogeneous shape into the extracellular space [1]. These vesicles that are often referred to as microparticles and sometimes also as

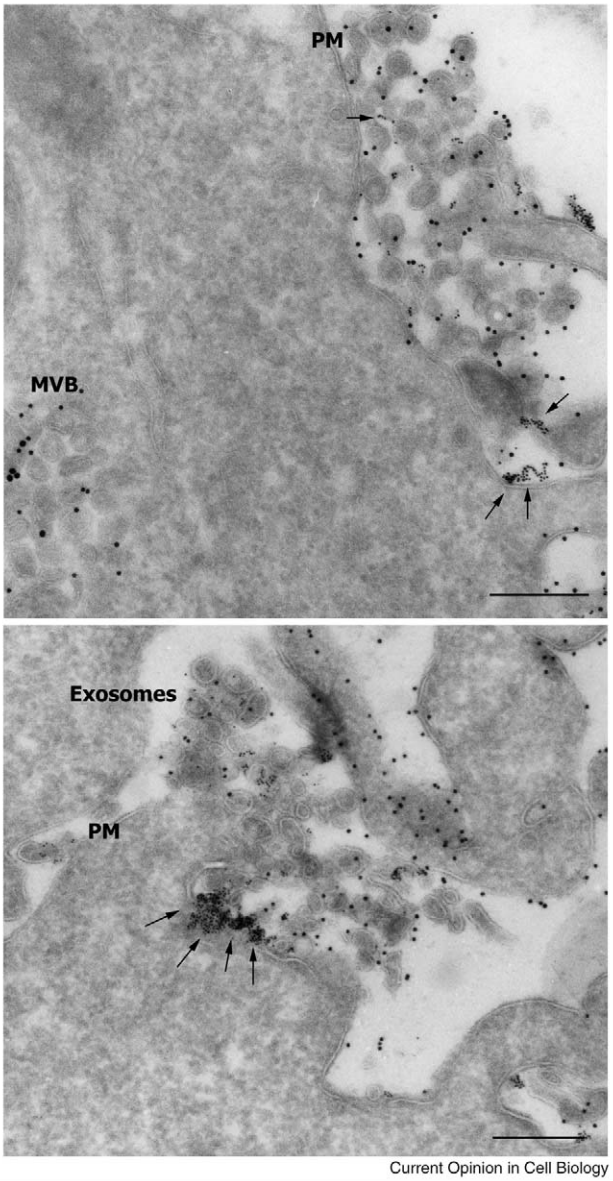
microvesicles, are generated by shedding from the plasma membrane. However, there are also vesicles with the typical size of exosomes (50–100 nm) that may bud from the plasma membrane [15]. The variety of extracellular vesicles released by different cell types has led to some confusion regarding the nomenclature. The endosomal origin is a commonly used criterion to define exosomes, while the shedding from the plasma membrane is applied to specify microvesicles. Since the experimental procedures used to purify extracellular membrane only allow a classification based on the size, density and the morphology and not on the origin of the vesicles, we propose to use the term microvesicles or microparticles for vesicles of heterogeneous size and shape (in general larger than >100 nm) and exosomes for vesicles of a size between 40 and 100 nm that carry the typical exosomal marker proteins. For example, the extracellular membrane particles containing the stem cell marker prominin-1 that are released from the midbody and the primary cilium of neural progenitor cells are 600 nm in size (diameter) and do not contain exosomal marker proteins and therefore classify as microparticles [16].

Exosome biogenesis

Exosomes correspond to the intraluminal vesicles (ILVs) of endosomal MVBs that fuse with the cell surface in an exocytic manner [3•,4,5]. Thus the biogenesis of exosomes is thought to occur within MVBs. Fusion profiles of MVBs with the plasma membrane have been observed by electron microscopy and purified exosomes are enriched in proteins that are found in ILVs. The best evidence that ILVs of MVBs are indeed released as exosomes derives from 'morphological' pulse-chase experiments, in which a label was allowed to internalize into a cell, and was later found to be released together with exosomes (Figure 1) [17].

Recent studies indicate that the molecular machinery that drives the formation of ILVs is more diverse than previously recognized. In general, the generation of ILVs involves the lateral segregation of the cargo within the limiting membrane of an endosome, followed by the formation of an inwardly budding vesicle and the release of the vesicle into its lumen. The role of the ESCRT (endosomal sorting complex required for transport) machinery in this process is well appreciated [18,19]. The ESCRT-0, -I and -II complexes recognize and sequester ubiquitinated proteins in the endosomal membrane, whereas the ESCRT-III complex seems to be responsible for membrane budding. While the ESCRT proteins, which were first identified in yeast genetic screens for vacuolar protein sorting (vps) mutants, are required for the targeting of membrane for lysosomal degradation, the function of the ESCRT machinery in the formation of ILVs that are further secreted as exosomes is less clear. There is still no clear evidence whether the sorting of selected exosomal cargo requires ESCRT function. Proteomic analyses of purified exosomes from various cell types show an enrichment of different ESCRT components and ubiquitinated proteins [20,21]. Moreover, the expression of the Nedd4 family interacting-protein 1 is associated with elevated levels of

Figure 1



Exosomes are released upon exocytic fusion of MVBs with the plasma membrane.
B-EBV transformed B lymphocytes were allowed to internalize 5 nm BSAG particles for 10 min and chased for 20 min (upper panel) or 50 min (lower panel) in the absence of BSAG. Ultrathin cryosections were immunogold labeled for MHC class II (Protein A gold 10 nm). The fusion profile (arrows) is defined by the presence of externalized BSAG that had previously been endocytosed. In addition to BSAG, the exocytic profile contains MHC class II-labeled vesicles (exosomes). PM: plasma membrane; MVB: multivesicular body. Bars: 200 nm.

protein ubiquitination in exosomes [22], and Alix, a protein that associates with the ESCRT machinery, is required for the sorting of the transferrin receptor into exosomes [23]. These observations lead to the hypothesis that ESCRT function could be required for sorting of exosomal cargo.

However, recent studies have provided evidence that some exosomal proteins are released in an ESCRT-independent manner [14•,24•]. One of these pathways requires the lipid, ceramide [14•,25,26]. Ceramide has many structural and physical properties that may facilitate vesicle biogenesis. Ceramides are known to induce lateral phase separation and domain formation in model membranes [27]. In addition, the cone-shaped structure of ceramide may induce spontaneous negative curvature in the membrane bilayer promoting membrane invagination. Proteins, such as tetraspanins, may partition into these domains. It is also possible that tetraspanins are part of the sorting system as they are known to form oligomers (tetraspanin webs) by interacting with other tetraspanins and also with a variety of transmembrane and cytosolic proteins [28].

Indeed, there is increasing evidence that the clustering of exosomal cargo is an important sorting determinant. For example, antibody-induced crosslinking of the transferrin receptor in reticulocytes [29], the MHC-II in lymphocytes [30] or CD43 in Jurkat T cells [24•] enhances their secretion in association with exosomes. Furthermore, the addition of multiple homo-oligomerization domains to an acylated reporter protein increases its exosomal release [24•]. The oligomerization of exosomal components may lead to recruitment and stabilization of exosomal membrane domains. If these grow beyond a critical size, possibly domain-induced budding is triggered and vesicles are formed after fission has occurred at the domain boundaries [31]. In fact, exosomes are enriched in raft-lipids and one driving force in forming exosomes may, thus, be the line-tension driven budding between liquid-ordered and disordered domain boundaries. Consequently, the function of the ESCRT-machinery to recruit cargo and to deform the membrane does not seem to be required in this pathway. There are some examples of proteins that may use such a pathway. The melanosomal protein (Pmel17) is sorted to the intraluminal vesicles of mutlivesicular endosomes by a lumenal domain-dependent and an ESCRT-independent pathway [32•]. Furthermore, the exosomal release of CD63, CD82 and the proteolipid protein (PLP) was not affected after inhibition of class E VPS proteins [14•,24•]. In addition, sorting of MHC II into exosomes is, in contrast to its lysosomal targeting, independent of MHC II ubiquitination but rather correlates with its incorporation into tetraspanin-containing detergent-resistant membranes (W Stoorvogel, personal communication).

However, exosomes do not only vehicle proteins and lipids. There is increasing evidence that they also carry a large number of selected mRNAs and microRNAs [33••]. Although present in the cytosol these different RNA species are likely to be specifically packed into the exosomes by active sorting mechanisms as only a subset of transcripts was found. Since the ESCRT-II subunits are able to specifically bind mRNA [34], an involvement of the ESCRT-machinery in the sorting of RNA into exosomes is still an attractive possibility.

Vesicles with hallmarks of exosomes are not only formed in MVBs, but also at the plasma membrane in specific domains, which have been termed endosome-like domains by some authors [15]. These domains were first detected in Jurkat T cells and were shown to be enriched for exosomal and endosomal proteins such as CD81 and CD63. In addition, *N*-Rh-PE (1,2-dipalmitoyl-sn-glycero-3-phophoethanolamine-*N*-[lissamine rhodamine B sulfonyl]), a lipid that is normally internalized and released with exosomes, accumulated within these domains. By electron microscopy budding profiles were detected within these domains showing that they indeed serve as sites for exosome biogenesis. Moreover, the addition of acyl-chains to a reporter protein improved the targeting to the plasma membrane domains and enhanced exosome secretion [24•]. In some cells these plasma membrane domains are internally sequestered in an extensive network of interconnected sponge-like membrane [35,36].

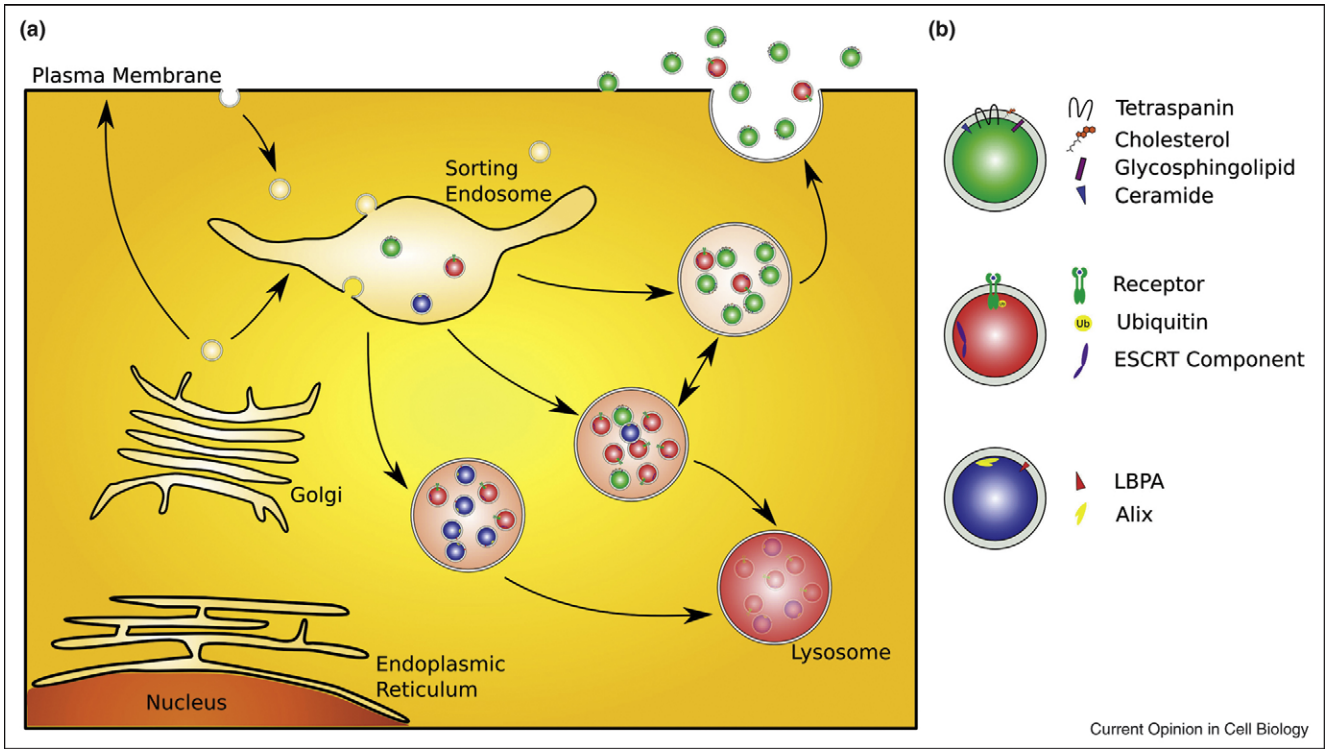
The relative abundance of these different pathways of exosome generation depends on the cell type and only further insights into the mechanisms of exosome biogenesis will allow an accurate classification.

MVB subpopulations

There is accumulating evidence that MVBs are more heterogeneous than previously appreciated. It is clear that there must be different kinds of MVBs – one class that eventually ends up in lysosomes and another class that fuses with the plasma membrane to release exosomes – but methods to separate these subpopulations are not available. Whether these MVBs contain intermixed populations of ILVs or specific set of ILVs destined either for the release as exosomes or for delivery to lysosoems is not known.

One first line of evidence for different subclasses of MVBs with different fates comes from studies using perfringolysin O to label cholesterol. Cholesterol positive and cholesterol negative MVBs coexisted in B lymphocytes and interestingly those enriched in cholesterol appeared to fuse with the cell surface to release exosomes [37]. More evidence for the existence of MVB subpopulations comes from a study that shows that EGF and its receptor traffic through a subpopulation of MVBs not containing the lipid bis(monoacylglycero)phosphate/lysobisphosphatidic acid

Figure 2



Model for sorting of cargo into different MVB subpopulations. (**a**) Different hypothetical MVB subclasses with distinct populations of ILVs (red, green and blue) are shown. The putative compositions of these ILVs are shown in the right panel. Whether the MVBs contain a mixture of different ILVs as depicted in the figure is not known. Sorting of cargo may already start within the biosynthetic pathway, at the plasma membrane or within an endosomal compartment. (**b**) At least three different subclasses of ILVs may coexist. The molecules shown represent a selection of protein and lipids that define different classes of ILVs.

(BMP/LBPA) [38]. BMP/LBPA tends to spontaneously form inwardly budding vesicles on liposomes in an Alix- and PH-dependent manner [39]. However, such vesicles do not appear to play a role in exosome formation, as mass spectrometry analysis of the lipid composition of exosomes from human B-cells revealed that BMP/LBPA is absent from exosomes [12]. Instead the BMP/LBPA containing ILVs are functionally implicated in the control of the back-fusion of ILVs with the limiting endosomal membrane and in the regulation of degradative processes within the lysosomal pathway [40,41].

Of note, concomitant depletion of four components of the ESCRT- machinery (Hrs, Tsg101, Vps22 and Vps24 components of ESCRT-0,I,II,III, respectively) blocked the sorting of the EGFR, but did not result in a complete inhibition of the generation of organelles with hallmarks of MVBs (H Stenmark, personal communication). Whether these MVBs represent a subclass of MVB, possibly with secretory properties is an interesting question.

Regulation of exosome secretion

Multiple pathways seem, thus, to operate on endosomal membranes and give rise to different populations MVBs and ILVs (Figure 2). An important goal is to understand if the different populations of MVBs emerge together to finally segregate from each other or if they originate independently. It would be equally important to be able to define the different types of MVBs not only on the basis of their morphology but also on their composition. These distinct MVBs require different mechanisms to regulate fusion with the target membrane. While the largest population of MVBs continuously fuse with lysosomes in most cells [42], the fusion events are prevented in some instances as MVBs can also house proteins for temporary storage. Little is known about the requirements and regulation of MVB fusion with the plasma membrane. As for lysosomal secretion [43], calcium ionophores stimulate exosome release in some cell types suggesting that intracellular calcium levels play a role in plasma membrane fusions event [44–46]. However the fusion machinery regulated by Ca^{2+} remains undefined. The small GTPase Rab11 and the citron kinase, a RhoA effector, may participate in the exocytic event [47,48]. A potential candidate that has been proposed to mediate fusion of MVBs with the plasma membrane is the V0-subunit of the V-ATPase. Independently of the V0-V1 ATPase complex, the V0-subunit plays a role in membrane fusion [49] and a V0-

ATPase mutant in *Caenorhabditis elegans* impairs fusion of MVBs with the apical membrane of the cuticles [50]. However, final evidence for its direct involvement in MVB fusion, and exosome secretion is still awaiting further studies in mammalian studies enabling a quantitative analysis of secreted exosomes.

There are also conditions, in which fusion of MVBs with the plasma membrane is prevented. For example, induction of autophagy inhibits exosome secretion and promotes the fusion of MVBs with autophagic vacuoles [51].

Exosome fate

Within the extracellular space and in biological fluids (e.g. urine, serum,) exosomes have been suggested to participate in different physiological and/or pathological processes [3•,4,5]. For some cells exosome secretion is a system to dispose unwanted proteins. Exosomal release instead of lysosomal processing might be of advantage to cells that have poor degradative capacities or are located towards a drainage system such as the tubules of the kidney or the gut [52]. Depending on their origin many different functions have been attributed to exosomes. For example platelets secrete exosomes after activation in order to regulate coagulation events [1]. Here, exosomes are used to potentiate a physiological response by providing a larger surface for a reaction. A role for exosomes in the migration of Dictyostelium cells by the secretion chemoattractant signals has recently been proposed [53•]. This study shows that migrating cells accumulate MVBs and secrete vesicles at the back of the cells. These vesicles may form extracellular tracks presumably involved in the formation of head to tail arrays of migrating cells. However, the most distinctive function of exosomes is to specifically interact with a target cell to regulate cell–cell communication. Their intrinsic features (for example sets of adhesion molecules) enable their specific targeting to a recipient cell and this has been particularly highlighted in the immune system (see reviews on this topic for a detailed discussion [4]), but also in communication between the intestinal epithelium and the mucosal immune system [54]. For example, professional antigen-presenting cells such as dendritic cells release exosomes that contain MHC and costimulatory molecules to stimulate T-cell activation. The intercellular adhesion molecule 1 (ICAM-1) and the leukocyte function – associated antigen-1 (LFA-1) are used for the capture of the exosomes at the cell surface [55–57]. In addition, the T-cell immunoglobulin – and mucin-domain-containing molecules (Tim-1 and -4) were recently identified as phosphatidylserine receptors that are able to bind exosomes via phosphatidylserine [58•].

Recent studies indicate that exosomes are not only specifically targeted to recipient cells to exchange proteins and lipids and/or to trigger downstream signalling events, but are also able to deliver genetic material [59]. Mast cells secrete exosomes that contain mRNA from approximately 1300 genes and >100 different microRNAs [33••]. The transfer of exosomes to a donor cell showed that the mRNAs were active as their translational was initiated in the recipient cell. Similarly, glioblastoma cells release microvesicles/exosomes containing mRNA, microRNA and angiogenic proteins [60]. When internalized into human brain microvascular endothelial cells, angiogenesis was stimulated. These provocative studies suggest that exosomes have virus-like properties being able to transfer nucleic acid into a recipient cell. While it is well established how virus fuse with their host membrane to release their genome into the cytosol, it is not known whether exosomes are able to do this. Given that exosomes are able to be endocytosed into the endosomal system of recipient cells, the most attractive hypothesis is that, once internalized, exosomes could fuse with the limiting membrane of endosomes to deliver their cytosolic content into the host cell cytoplasm. It is still possible that exosomes can directly fuse with the plasma membrane. Proteome analyses of exosomes have shown that the fusogenic protein, CD9, is abundantly expressed in exosomes pointing to one candidate molecule [61]. Clearly, our understanding of how exosomes are processed in recipient cells is only at the beginning. This is particularly relevant as exosomes might also play a role in spreading pathogens such as prions from one cell to the other [62]. The transfer of oncogenic activity by exosomes from tumour cells has also been described [63].

Conclusions

During the past few years, there has been a remarkable increase in the number of studies that aim to understand the biology of exosomes. Exosome secretion is more widely used by cells and organisms as previously appreciated and several studies highlight their implication in both physiological and pathological processes. Moreover, these small vehicles have appealing properties that can be exploited for therapeutical intervention [64] and as biomarkers present in biological fluids in pathological situations [6,7]. We are now at an early stage of understanding how exosomes are formed. The clustering and sorting of cargo at the limiting membrane of vacuolar domains of endosomes seems to be a driving force for the recruitment and the formation of the inward budding vesicles. Exosomes seem to comprise of a heterogeneous population of vesicles that can be generated by different mechanisms. So far exosomes are classified based on their size, density and molecular composition, but further progress in the mechanisms of exosome biogenesis and secretion will allow more accurate ways of discrimination in the future. The versatile means of origins and the specific molecular constituents reflect the various functions that have

been unravelled. The most provocative interpretation of exosomes is that they represent vesicular carries with virus-like properties that regulate gene regulation.

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Note added in proof

The papers refered to in the text as H Stenmark and W Stoorvogel personal communication have now been accepted for publication [65,66].

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