Proteomic analysis of early and late endosomes has been constrained by the limited purity of the endosomal fractions that can be achieved by biochemical methods. Here we briefly review endocytic pathways, and then introduce fractionation strategies that have been used to improve the purity of isolated endosomes. In addition, we describe innovative proteomics analysis methods that have been shown to partially circumvent the limitations found in the enrichment steps.

**BACKGROUND**

Polar macromolecules and solutes are internalized in cells through a process called endocytosis. Several routes (clatherin-mediated endocytosis, micropinocytosis, phagocytosis, and caveolae) are used to deliver the engulfed material to a morphologically distinct compartment called the early endosome, which is the major sorting station in the endocytic pathway. In a tightly regulated process, constant budding and fusion events allow membrane and cargo flow between early endosomes and recycling endosomes, plasma membrane, multivesicular bodies (MVBs), late endosomes, and the trans-Golgi network (TGN) (Fig. 1).

The recycling route from the early endosome to the plasma membrane seems to be set by default, with no specific motif being assigned to the transported cargoes. Several proteins including Rab4, Rab5, Rab11, and the SNARE protein cellubrevin are present in these tubular recycling vesicles (Gruenberg 2001). Transport from early to late endosomes is mediated by large invaginated structures called MVBs. These structures are transported via microtubules to the perinuclear region, where they fuse with late endosomes in a step mediated by conventional docking/fusing machinery (Gu and Gruenberg 1999; Chen and Scheller 2001; Gruenberg 2001). MVBs differ from late endosomes in that their limiting membrane does not contain high amounts of LAMP1. It is still unclear whether MVBs are actually defined transport intermediates or whether, by a maturation process, they can give rise to late endosomal structures.

Late endosomes are morphologically complex vesicles with tubular cisternae and invaginated regions. The limiting membrane contains high amounts of LAMP1 and MLN64, and internal vesicles are enriched in CD63 and lysobisphosphatidic acid, a lipid believed to be synthesized in situ and that, because of its shape, facilitates the formation of the intraorganelle vesicles (Gruenberg 2001). Certain proteins and receptors (e.g., the epidermal growth factor receptor) are monoubiquitinated before partitioning into the internal invaginations of the late endosome (Amerik and Hochstrasser 2004). This process sequesters the cargo from cytoplasmic interacting partners and renders them accessible to the lysosomal enzymes. However, internal membranes also accumulate mannose-6-phosphate receptor, CD63, tetraspanins, and MHC-II receptor, all examples of in-transit proteins that are not
subjected to degradation but are cycled back to the limiting membrane via back fusion (Griffiths et al. 1988; Mobius et al. 2003). It has been proposed that lipid heterogeneity of internal membrane vesicles of the late endosome could, at least in part, explain the differences observed (Kobayashi et al. 2002).

Finally, late endosomes exchange content in a rapid manner with lysosomes, the organelles responsible for the degradation of endocytosed cargo. Late endosomes and lysosomes are both acidic, have the same glycolipids on their membranes, and even share lysosomal enzymes. In fact, the only distinct feature of lysosomes is the lack of specific late endosomal markers like Rab7.

**THE ENDOesome PROTEOME**

Recent findings have showed transient interactions between different endosomal organelles (Duclos et al. 2003) and have also highlighted that different Rab proteins, frequently taken as organelle markers, can be present on the same endosome (Sonnichsen et al. 2000). These data, together with evidence supporting the existence of different subpopulations of early endosomes (or different regions and membrane domains within each early or late endosome) (Sonnichsen et al. 2000; Gruenberg 2001, 2003; de Renzis et al. 2002; Russell et al. 2006; van der Goot and Gruenberg 2006), clearly indicate that the complexity of the endocytic organelles and their biogenesis is far from being understood. The isolation of endocytic organelles and the charting of the individual organelle proteomes are therefore seen as fundamental tools for understanding the regulation of endocytic transport and its intrinsic dependency on intracellular signaling events.

From a proteomics perspective, whole-cell or tissue analysis remains in most cases too complex to be determined accurately. From the far more than ten thousands of estimated gene products present in a given human cell, the presence of splice variants and posttranslational modifications that may vary in time according to stimulus increases severalfold the number of protein species to be analyzed. Furthermore, the large variance in protein expression, with low-abundance proteins being masked by high-abundance ones, strongly hinders the correct charting of regulatory proteins like kinases and receptors (Jung et al. 2000; Brunet et al. 2003; Huber 2003).
The organization of the eukaryotic cell into subcompartments (organelles) provides a unique opportunity to reduce sample complexity and at the same time increase knowledge about identified proteins and their subcellular localization. Over the last decades, several methods have been used to isolate intracellular organelles, from immunoisolation to electromigratory techniques, fluorescence-activated organelle sorting, and the commonly used density gradient centrifugation.

With the exception of phagocytosis, the organelle proteome analysis of the remaining endocytic routes has been limited by the reduced purity of the fractions that are obtained by such methods. In addition, the constant flow of proteins from and to a given organelle leads to proteome changes that are time- and signaling-dependent. Such limitations make it difficult to determine if a given protein is a contaminant or a protein transiently associating with the purified organelle (Huber et al. 2003).

ADVANCES IN FRACTIONATION TECHNIQUES

Despite the difficulties, refinements have been made to subcellular fractionation methods that have improved the purity of the purified organelles. As an example, endocytic organelles can be loaded with perturbants that alter the density of a specific compartment thereby improving purification results. Opsonized latex beads are perturbants that have been used as tools to purify enriched phagosomal fractions from diverse species and under diverse conditions for nearly two decades. The initial screens performed by Desjardins et al. using two-dimensional gel electrophoresis (Desjardins et al. 1994a,b) have been complemented with more advanced liquid chromatography with tandem mass spectrometry (LC–MS–MS) methods to chart the phagosome proteome from cells as diverse as mouse macrophages, embryonic hematocytes from *Drosophila melanogaster*, human neutrophils and dendritic cells, and *Entamoeba histolytica* (Garin et al. 2001; Burlak et al. 2006; Okada et al. 2006; Rogers and Foster 2007; Stuart et al. 2007; Lee et al. 2010; Buschow et al. 2012; He et al. 2012). Such bead internalization methods have only recently been adapted to the isolation of late and early endosomal fractions (Duclos et al. 2011). In 2009 Grimes and coworkers reported a new fractionation approach enabling the separation of different types of early endosomes (McCaffrey et al. 2009). In brief, the size heterogeneity of the early endosomes is used as a tool to separate them: Organelles are separated by velocity gradient sedimentation (size) followed by density gradient centrifugation. The authors also found that three different kinds of receptors internalize into distinct early endosomes further extending the model of signaling endosomes.

We have used some of these techniques to devise a homogenization protocol (see Protocol: Homogenization of Mammalian Cells [de Araújo et al. 2014a]) and density gradient centrifugation procedures specifically designed for the isolation of endocytic organelles (see Protocol: Isolation of Early and Late Endosomes by Density Gradient Centrifugation [de Araújo et al. 2014b] and Protocol: Isolation of Macrophage Early and Late Endosomes by Latex Beads Internalization and Density Gradient Centrifugation [Lamberti et al. 2014]). These protocols can be easily combined with the quantitative proteomics methods described below.

QUANTITATIVE PROTEOMIC METHODS

The advent of quantitative organelle proteomics has fulfilled its promise of delivering a more detailed organelle proteome. The proteome increased in size, but it did not necessarily become more reliable. To circumvent the high rate of false positives delivered, two recent analysis methods have been introduced. Localization of organelle proteins by isotope tagging is based on iTRAQ technology (Dunkley et al. 2004; Sadowski et al. 2006; Trotter et al. 2010). In brief, enriched organelle fractions obtained by gradient centrifugation are iTRAQ labeled and MS–MS analyzed. This approach determines protein distribution and abundance in different fractions and is particularly useful in the analysis of stimulus-dependent localization changes. Protein correlation profiling (PCP) is based on the generation of mass spectrometry intensity profiles of bona fide organelle markers on a
density centrifugation gradient. The identification of novel organelle proteins is then based on the comparison between the profile of the new proteins and that of specific organelle markers (Foster et al. 2006; Wiese et al. 2007; Dengjel et al. 2010). The main advantage of PCF is the reduction of false positive hits.

SUMMARY

The organelle proteomics studies performed to date have led to a better understanding of the molecular mechanisms involved in the regulation of the function of various organelles. Nevertheless, we are still far from delivering a detailed, reliable, time- and stimulus-dependent endosomal organelle proteome. Limitations in the preparative enrichment of the endosomal organelle fraction remain a serious challenge and innovative approaches are urgently needed.

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