A Proteomics Approach to Membrane Trafficking[1]

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Membrane trafficking, including that of integral membrane proteins as well as peripherally associated proteins, appears to be a vital process common to all eukaryotes. An important element of membrane trafficking is to determine the protein composition of the various endomembrane compartments. A major issue with such a compositional analysis is the difficulty of having to distinguish between resident components involved in specific tasks and the proteins that are in transit through the endomembrane system. Examples of resident proteins include components of the SNARE complex used to target membrane vesicles to different locations in the cell. In the case of functionally important residents, one would expect such proteins to have a fairly precise subcellular localization. In the case of proteins ''passing through'' an endosomal compartment en route to a final destination, one would expect to find the proteins colocalizing with many membrane compartments.

As is evident from several *Update* articles in this issue, ambiguity exists when employing cytological techniques to identify specific endomembrane compartments, while markers identified based on homology may behave differently in plant cells. Therefore, a proteomics approach based on proteins that would traffic through various parts of the endomembrane system, such as plasma membrane (PM) receptors, would be a welcome addition to membrane-trafficking studies. PM receptors are highly dependent on correct trafficking for their eventual localization, their biological function, and finally their degradation, while recent evidence suggests that endocytosis of PM receptors is an integral part of their biological function.

In this review, first, a short update on endocytosis and endosomal trafficking in Arabidopsis (*Arabidopsis thaliana*) is provided. In this section, we emphasize trafficking of PM receptors as a proteomics tool by looking at how the PM receptors traffic in a time-dependent fashion in order to determine the relationship between different endosomal compartments. Second, we describe the recent progress in advanced proteomics techniques such as localization of organelle proteins by isotope tagging (LOPIT), by which proteins are assigned to different endosomal compartments.

ENDOCYTOSIS

The importance of endocytosis of membrane receptors is discussed in the *Update* by Geldner and Robatzek (2008) in this issue and will only be briefly recapitulated here. Therefore, it would be of great importance to the field of plant receptor-mediated signaling to have a view of the protein composition of early sorting and late endosomal compartments. Only a few of these compartments have been identified with confidence. In animal cells, ligand-induced receptor endocytosis modulates sensitivity (''receptor down-regulation'') to ambient ligand concentration. Endocytosed receptors are sorted in early/sorting endosomes for either recycling via the endocytic recycling compartment or further internalization and eventual degradation in lysosomes (Maxfield and McGraw, 2004; Murphy et al., 2005). Some receptors, such as EGF (Wang et al., 2002) and PDGF (Wang et al., 2004), carry out signaling after having been endocytosed, and active endocytosed receptor kinases can also be inactivated by phosphatase activity (Haj et al., 2002). However, the process of endocytosis may have undergone evolutionary adaptive changes in the plant lineage: although the Arabidopsis genome encodes homologues of animal or yeast components of the endocytic machinery, the plant family members are often modified such that clear one-to-one orthologues are difficult to identify (Jürgens and Geldner, 2002). For example, the Arabidopsis homologues of the animal early endosomal marker Rab5, the RabF GTPases ARA6 and ARA7, appear to localize to different yet ill-defined endosomal compartments other than the early endosome (Lee et al., 2004; Ueda et al., 2004; Dettmer et al., 2006). Also, the Arabidopsis ARF-GEF GNOM is involved in endosomal recycling of PM proteins, whereas its closest yeast and animal homologues act at the Golgi stacks (Geldner et al., 2003). Recent evidence suggests that internalization and recycling or degradation of specific PM-localized proteins indeed occur in plant cells. For example, the boron transporter BOR1 accumulates at the PM at a low external concentration of boron but is internalized and degraded in response to high external concentration of

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boron (Takano et al., 2005). Ligand-dependent receptor-mediated endocytosis was recently demonstrated in plant cells for the flagellin receptor (Robatzek et al., 2006; Chinchilla et al., 2007), but as yet no evidence has been provided for an interaction between this receptor and the endocytic machinery, such as clathrin-associated proteins or adaptor protein (AP) complexes. Other examples are the receptor-like protein LeEIX2, which seems to be internalized before signaling takes place (Ron and Avni, 2004), the rapid internalization of the ACR4 receptor (Gifford et al., 2005), and the BRI1 receptor that appears to be able to continue to activate downstream signaling processes after endocytosis (Geldner et al., 2003). Limited information about the clathrin-based internalization machinery is known, and multivesicular bodies have been identified as both prevacuolar and endocytic organelles, but a definitive classification of endosomes into early/sorting, recycling, and late compartments has not yet been possible (Tse et al., 2004). For example, the trans-Golgi network may not only be a station (intermediate location on the secretory pathway) but might also correspond to an early endosome (Dettmer et al., 2006). Likewise, recycling endosomes have been defined functionally as PIN1-accumulating brefeldin A (BFA) compartments, depending on the BFA sensitivity of the ARF-GEF GNOM (Geldner et al., 2003). However, BFA treatment, which facilitates the immunological detection of endosomal markers, leads to the aggregation of several endosomal compartments and thereby precludes their spatial resolution by confocal laser-scanning microscopy and GFP technology (Dettmer et al., 2006). Recently, one of the first pieces of evidence was obtained for the existence of sorting endosomes in Arabidopsis roots, where the sorting nexin AtSNX1 was found to overlap with BP80, a marker for the prevacuolar complex. Functional evidence employing drugs such as wortmannin, which blocks endocytosis from the PM to the early endosome trans-Golgi network, suggests that indeed the AtSNX1-marked compartments can participate in sorting receptors, such as the main brassinolide-perceiving receptor BRI1, and are part of a multivesicular sorting endosome/prevacuolar complex/multivesicular body network (Jaillais et al., 2008).

An attractive alternative to identifying endosomal compartments by attempting proteomics analysis of successive stations on the endocytic pathway (which will be discussed in the next section) would be to use well-characterized model receptors. The well-characterized trafficking pathway of the human transferrin receptor hTfR (Mellman, 1996) has been investigated in Arabidopsis protoplasts and tested for its functionality and trafficking properties (Ortiz-Zapater et al., 2006). The internalized hTfR localized to putative endosomal compartments, as judged by its colocalization with the Rab GTPase ARA7. However, as in mammalian cells, a small fraction of the receptor is also found at the PM, where it mediates the binding and internalization of its natural ligand transferrin, Tfn. Internalized Tfn colocalizes with the hTfR as well as with FM4-64 internalized for 15 min, suggesting that both Tfn and hTfR enter an endosomal (ARA7, FM4-64 positive) compartment. Treatment with tyrphostin A23, which inhibits the interaction between the YTRF endocytosis motif in the hTfR cytosolic tail and the μ2 subunit of the AP-2 complex (Aniento and Robinson, 2005), blocks Tfn internalization and redistributes most of the hTfR to the PM. This suggested that hTfR has the same trafficking properties in Arabidopsis protoplasts as in mammalian cells and is an excellent marker to isolate and characterize early and recycling endosomal compartments in plant cells. Moreover, hTfR interacts with a μ-adaptin subunit from Arabidopsis cytosol, and this interaction is prevented by tyrphostin A23. These data provided the first tangible link between an internalized receptor and elements of the clathrin internalization machinery in plant cells.

The main brassinolide (BR) receptor in plants is BRI1 (Li and Chory, 1997). BR binds to the extracellular domain of the BRI1 receptor (Kinoshita et al., 2005), but ligand-mediated endocytosis has not been reported so far. BRI1 acts in a complex with the non-ligand-binding coreceptor BAK1 (Wang et al., 2005b) that is the third member of the SERK family of receptors. The PP2C phosphatase KAPP enhances internalization of the SERK1 receptor (Shah et al., 2002), and both the SERK3/BAK1 and SERK1 coreceptors enhance endocytosis of BRI1 into FM4-64-labeled endosomal complexes (Russinova et al., 2004). Genetic and biochemical evidence suggests that SERK1 and SERK3/BAK1 act as redundant coreceptors in BR signaling and may also provide a means to confer specificity to overall BR signaling (Karlova et al., 2006). In this last study, the approach to use the tagged membrane receptor SERK1 to identify associated proteins provided evidence that components of different membrane compartments during receptor trafficking can be identified using a proteomics approach. Karlova et al. (2006) not only identified the receptors BRI1 and SERK3/BAK1, which reflected the complex at the PM and during endocytosis, but also the CDC48 protein, residing in the ER (Aker et al., 2006; see Aker and de Vries, 2008). So far, only a few potential constituents of endosomal compartments such as ARFs have been identified using this proteomics approach (R. Karlova and S.C. de Vries, unpublished data), suggesting that the amount of PM receptors residing in endosomes may be relatively low compared with the ER or PM. Clearly, enrichment of endosomal compartments with the PM receptors present as cargo is first required.

Study of Organelle Proteomes Using Quantitative Proteomics

While using well-characterized receptor models could lead to the identification of new endosomal compartments, sophisticated proteomics approaches are required to study the intracellular trafficking of proteins between endocytic compartments. Identifica-

tion of the subproteome of the various subcompartments at different time points upon a stimulus is thus desirable. However, this most basic form of organelle proteomics (i.e. isolation of a compartment of interest; Steen and Mann, 2004) is not trivial. Although many strategies exist for enriching specific organelles (Huber et al., 2003; Brunet et al., 2004), the problem of contamination arises, since many subcompartments share similar physicochemical properties. The degree of contamination obviously differs among compartments and the chosen purification method. The proteome is also highly dynamic, with proteins trafficking from one compartment to the other, signaling pathways that share common routes (Dettmer et al., 2006), proteins that are continuously recycled, and proteins that are simply present in multiple compartments at the same time. Proteins can be posttranslationally modified and may also exist as differentially spliced variants that may differ in spatial distribution pattern. In short, it is a challenge to distinguish a true resident from a contaminant.

To overcome these difficulties, one approach is to take the proteomes of several subcompartments into account after incubation of the samples at 4°C and subsequent treatment with a ligand, assuming an overall steady-state protein distribution pattern. Subsequently, many of these steady-state distribution patterns at multiple time points will reveal protein dynamics. The basis of a method to investigate the proteomes of several organelles at the same time was noted by de Duve (1971). He suggested that a true resident had a characteristic distribution pattern within a gradient where analytical centrifugation was applied and that it was not restricted to a single fraction. In this way, the location of a protein of unknown residency can be identified by matching to specific marker proteins. In order to accurately map distribution patterns in a high-throughput manner, modern quantitative proteomics technologies such as tandem mass spectrometry (MS/MS) are routinely used on complex samples of peptides obtained after proteolytic digestion. Peptide mixtures are usually simplified by multidimensional separation before measurement by liquid chromatography-MS/MS (Washburn et al., 2001). These methods can be used in a quantitative manner by coupling them with the incorporation of differential stable isotopes or by label-free quantification. Measuring the peptide peak intensities (Andersen et al., 2003; Foster et al., 2006; Wiese

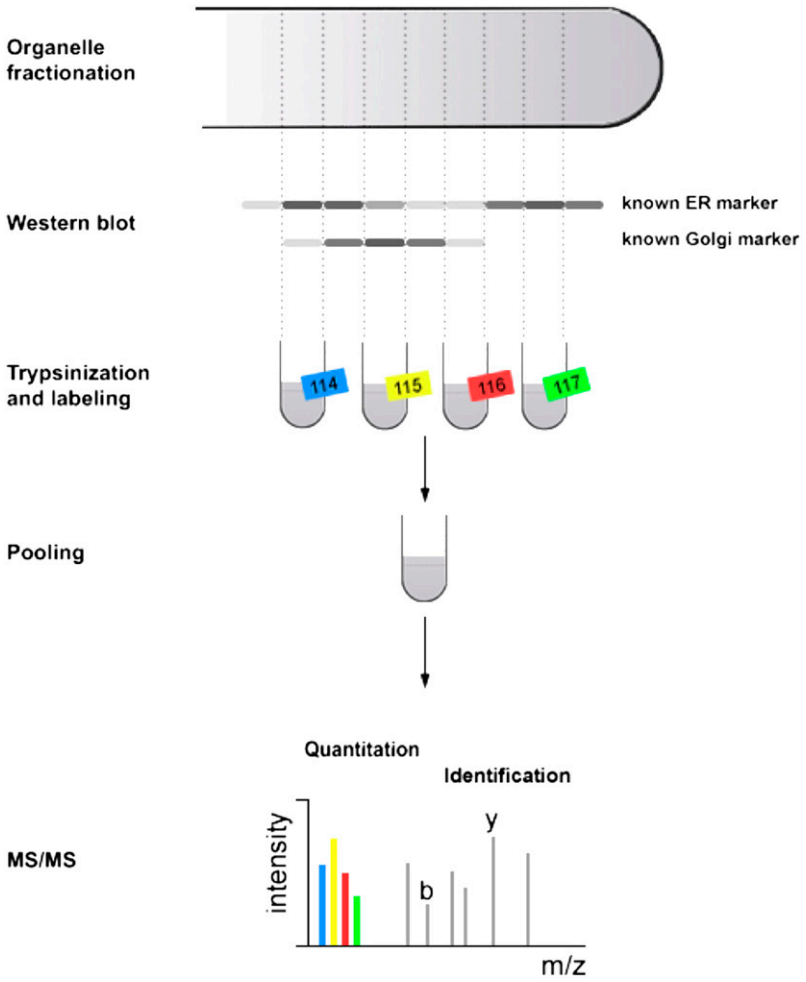

Figure 1. LOPIT schema. Many organelles can only be partially purified and are contaminated by other organelles with similar physicochemical characteristics, which could lead to mislocalization. LOPIT negates the need to obtain a pure organelle preparation, since this approach is dependent only on the enrichment of proteins along a gradient. The localization of membrane proteins is achieved after comparison of their enrichment patterns with those of membrane proteins already known to reside in a certain organelle. This is based on the assumption that proteins that belong to the same subcompartment have similar distribution patterns, whereas contaminant proteins have not. (Adapted by permission from Macmillan Publishers [Sadowski et al., 2006].)

et al., 2007) and counting the number of MS/MS spectra (Gilchrist et al., 2006; Kislinger et al., 2006) are both label-free methods. However, quantification is more challenging and is highly dependent on the reproducibility of peptide generation, chromatography, and ionization efficiency. Subtle distinction between proteins, and hence the confident assignment of proteins to specific organelles, might be lost due to the variation that occurs among the experiments. For these reasons, the utility of label-free methods is a matter of debate, although large proteome catalogues were obtained using these methods.

Both in vivo and in vitro labeling methods have been established to obtain improved reproducibility, since the samples are already pooled in an early experimental phase and thus share much of the experimental variance. Stable isotope labeling of amino acids in cell culture is an in vivo labeling method using cells grown in the presence of a heavy or a light isotope, after which the cells are subsequently pooled and the proteins are isolated, digested, and measured by liquid chromatography-MS/MS (Andersen et al., 2005; Rogers and Foster, 2007). The ratio between the isotopes gives information about the relative protein quantities in both samples. However, when dealing with intact plants under normal physiological conditions rather then liquid-grown seedlings (Wang et al., 2005a) or cell cultures, in vivo labeling may not always be possible. In vitro labeling of peptides is currently done using a differential isotope labeling method called iTRAQ (for amine-reactive reagents for relative and absolute quantitation; Dunkley et al., 2004; Ross et al., 2004). This approach involves labeling of peptides generated by proteolysis with four tags that label via primary amine groups in every peptide generated upon proteolysis. The tags are isobaric, so that peptides that are differentially tagged appear as a single precursor peak. Upon MS/MS, the iTRAQ tags release characteristic reporter ions providing very accurate quantification.

Assignment of Genuine Residents of Proteins to Endocytic Compartments Using LOPIT

LOPIT is an example of a technique that uses the iTRAQ technology (Dunkley et al., 2004, 2006; Sadowski et al., 2006). This technique analyzes the distribution of organellar proteins along a self-generating iodixanol density gradient. This is achieved by selecting four or more fractions enriched with different organelles, verified by western blotting, and analyzing protein distribution by measuring their relative abundance using iTRAQ reagents and MS/MS (Fig. 1). The clustering of proteins and the final assignment of proteins to organelles is achieved using multivariate statistical techniques like principal component analysis and partial least squares-discriminant analysis (Fig. 2). LOPIT has a low proportion of false positives and enables the study of several organelles in parallel. In a recent study, the application of LOPIT led to the identification of 689 organellar membrane proteins residing in the ER, the Golgi apparatus, the vacuole, and the PM of Arabidopsis (Dunkley et al., 2006). For comparison, before the LOPIT analysis, fewer than 30 proteins were assigned to either one of the major endomembrane compartments, while another 120 only had a predicted location. A surprisingly large number of ER-located proteins may reflect the complexity of the trafficking processes that take place inside these compartments. Proteins that are involved in translocation, folding, proteolysis, and glycosylation are abundant, as are proteins involved in cytochrome P450 machinery, SNAREs, and the COPI- and COPII-mediated transport between the ER and Golgi. In the

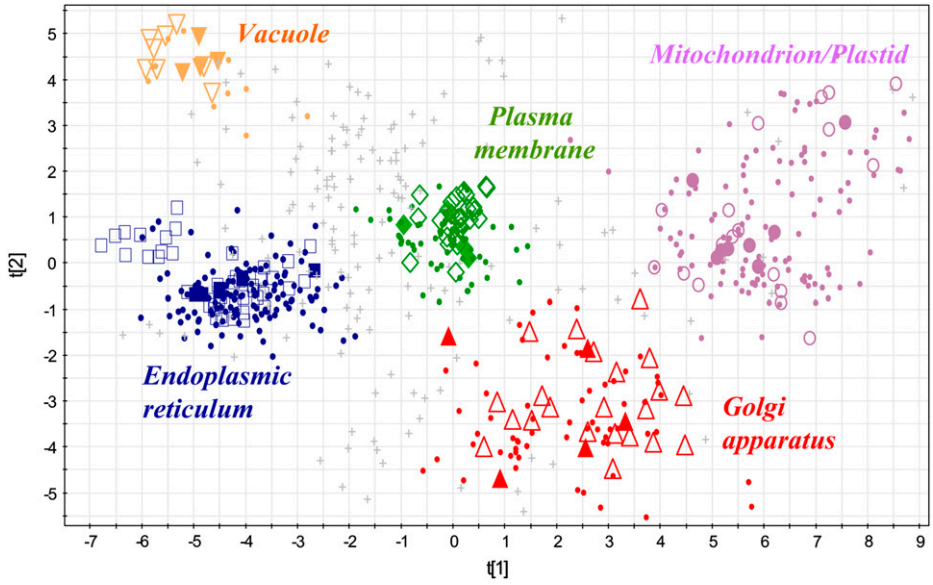

Figure 2. Principal component analysis of a LOPIT study. Typical results of a LOPIT experiment involving subcellular localization of Arabidopsis membrane proteins. Closed symbols, proteins with known localization; open symbols, proteins with predicted localization; small dots, proteins without prior localization and/or prediction and now assigned to an organelle by LOPIT; crosses, proteins that could not be assigned to an organelle. (Adapted by permission from *Proceedings of the National Academy of Sciences USA* [Dunkley et al., 2006].)

Golgi, the expected sets of glycosyl transferases, as well as many members of the EMP70 family and a previously unknown group of putative methyl transferases, were identified. Collectively, these data confirm and extend insights into the role of the endomembrane system. An orthogonal validation study using 22 GFP-fused proteins showed a low false-positive rate. The LOPIT results, therefore, provide a snapshot of the cell interior that shows the distribution of the proteins within these organelles at that given time point. In a recent study, additional proteins were identified compared with the study by Dunkley et al. (2006), and 193 proteins could be cross-referenced and thus were additionally validated (Sadowski et al., 2008).

OUTLOOK

LOPIT has the potential to look at the protein content and distribution of all intracellular compartments. Therefore, the application of LOPIT to investigate the protein content of endocytosed vesicles and endosomes is a promising strategy, especially since the protein composition of the major organellar compartments in Arabidopsis is now available as a point of reference. Given that the very goal of endosomes is trafficking, the underlying processes are most likely highly dynamic. LOPIT has the potential to discriminate between full-time residents and proteins in transit. Therefore, using LOPIT to track the synthesis, sorting, and endocytosis of receptors as marker proteins will be a major challenge to complete but should help us understand the various stations and compartments where interaction with the resident machinery occurs.

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