Diversity in unconventional protein secretion

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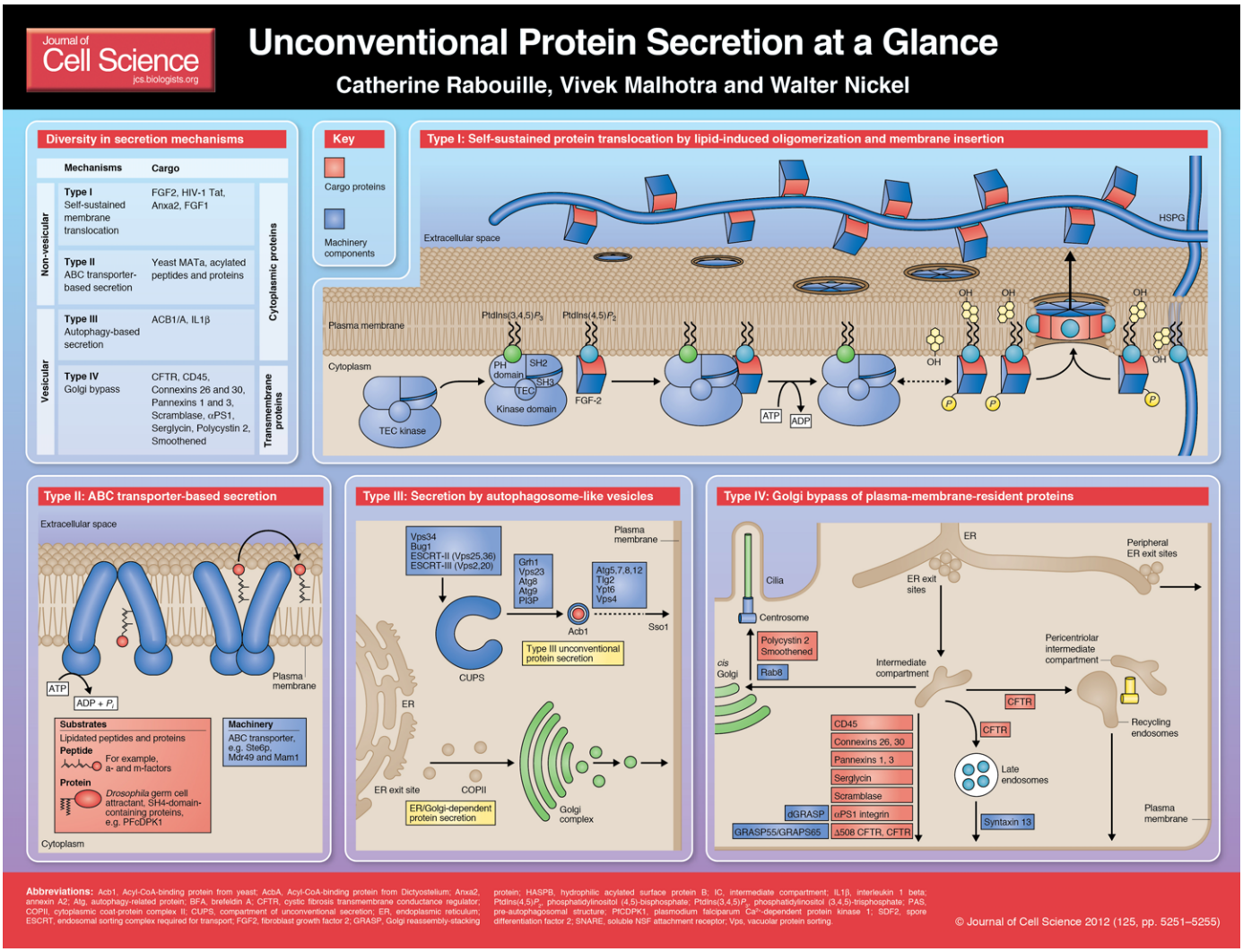
Eukaryotic cells use the endoplasmic reticulum (ER)-to-Golgi membrane pathway for the secretion of the vast majority of secretory proteins. This process is initiated by signal-peptide-dependent protein translocation into the lumen of the ER followed by vesicular transport of secretory cargo to the Golgi membranes and thence to the cell surface. However, a substantial number of proteins that lack a signal peptide are secreted without entering the conventional ER-to-Golgi pathway of protein secretion. These proteins are generally involved in cell survival, immune surveillance and tissue organization and, therefore, are of fundamental importance. Recent studies have uncovered the existence of four principal types of unconventional protein secretion that can be distinguished into non-vesicular and vesicular pathways (see Poster). The non-vesicular pathways encompass self-sustained protein translocation across plasma membranes (type I) and ABC-transporter-based secretion (type II). Vesicular pathways are characterized by autophagy-based secretion (type III) and proteins that bypass the Golgi complex (hereafter referred to as the Golgi bypass) for trafficking to the plasma membrane (type IV). Importantly, types I, II and III are pathways that mediate unconventional secretion of cytoplasmic proteins – that, in case of type II, are acylated. By contrast, all known examples of the type IV pathway are integral membrane proteins.

Type I – self-sustained protein translocation through lipid-induced oligomerization and membrane insertion

The type I of unconventional secretion refers to direct translocation of



cytoplasmic proteins across the plasma membrane without the involvement of vesicular intermediates (see Poster). The best studied example for this type of secretion is the release of fibroblast growth factor 2 (FGF2) (Nickel, 2011), which involves interaction of FGF2 with the phosphoinositide phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P_2) at the inner leaflet and with heparan sulfates at the outer leaflet of the plasma membrane (Schäfer et al., 2004; Zehe et al., 2006; Temmerman et al., 2008). The mechanism by which FGF2 traverses the plasma membrane depends on a fully folded conformation of FGF2 (Backhaus et al., 2004; Torrado et al., 2009), a key finding that argues against the participation of a classic protein-conducting channel at the level of the plasma membrane. Recently, membrane translocation of FGF2 was revealed to involve PtdIns(4,5)P_2-induced oligomerization at the inner membrane leaflet as well as insertion of FGF2 into the membrane (Steringer et al., 2012). This process is strongly stimulated by phosphorylation of FGF at tyrosine residue 82 through Tec kinase (Ebert et al., 2010). Reconstitution experiments – that were carried out to analyze transbilayer diffusion of membrane lipids by using model membranes and purified proteins – have established that PtdIns(4,5)P_2-dependent oligomerization and membrane insertion of FGF2 is triggered by the formation of a lipidic membrane pore with a putative toroidal architecture (Steringer et al., 2012). We speculate this structure is a ring-like FGF2 hexamer that is located in the center of the pore (see Poster), and believe that it represents a transient translocation intermediate in the process of FGF2 secretion that is rapidly altered when FGF2 is extracellularly trapped by binding to heparan sulfate proteoglycans (see Poster).

In conclusion, membrane translocation of FGF2 is mediated by a self-sustained mechanism that is driven by PtdIns(4,5)P_2-dependent oligomerization and tyrosine phosphorylation of FGF2. This process generates an intermediate structure of FGF2 oligomers that open and stabilize a lipidic membrane pore without contact of the FGF2 oligomer to the inner hydrophobic core of the plasma membrane (see Poster) (Steringer et al., 2012). In this context, one may speculate that – thus far unidentified – membrane proteins exist that prevent a sustained breakdown of the membrane potential or a substantial loss of lipid asymmetry during membrane translocation of FGF2. Such a mechanism could explain how cells can tolerate a mechanism of translocation through the plasma membrane that involves transient disturbance of the regular lipid bilayer structure.

These observations indicate a mechanism of unconventional secretion of FGF2 that is based on (i) recruitment by acidic membrane lipids at the inner leaflet, (ii) oligomerization-induced membrane insertion and (iii) tyrosine phosphorylation. This mechanism might be relevant for other unconventionally secreted proteins, such as HIV-1 Tat (Rayne et al., 2010a; Rayne et al., 2010b), annexin A2 (Anxa2) (Deora et al., 2004), and FGF1 (Prudovsky et al., 2008). Specifically, these proteins are known to bind to acidic membrane lipids, including phosphoinositides (Tarantini et al., 1995; Rescher et al., 2004; Rayne et al., 2010b). Further, they appear to be able to traverse the plasma membrane (Prudovsky et al., 2002; Deora et al., 2004; Rayne et al., 2010a; Rayne et al., 2010b; Kirov et al., 2012) and, in case of HIV-1 Tat secretion, a direct requirement for PtdIns(4,5)P_2 has been demonstrated (Rayne et al., 2010b). Furthermore, translocation of Anxa2 to cell surfaces has been shown to depend on tyrosine phosphorylation (Deora et al., 2004). Finally, both Anxa2 and FGF1 have been found to engage in hetero-oligomeric complexes that – while interacting with acidic membrane lipids – are proposed to be important for translocation to cell surfaces (Prudovsky et al., 2008). Therefore, a sub-group of unconventionally secreted proteins seems to exist, whose translocation across plasma membranes depends on similar molecular requirements. We, therefore, propose to classify these processes as type I unconventional protein secretion.

Type II – ABC-transporter-based secretion

A second non-vesicular mechanism of unconventional protein secretion is mediated by ABC transporters (see Poster). This pathway appears to be used to translocate lipidated peptides and proteins across the plasma membrane of eukaryotic cells, an example is the yeast pheromone a-factor, a farnesylated peptide that is externalized by the ABC transporter Ste6p (McGrath and Varshavsky, 1989; Michaelis, 1993). Similarly, the farnesylated m-factor of *Schizosaccharomyces pombe* is exported by the ABC transporter Mam1 (Christensen et al., 1997). Intriguingly – mediated by the ABC transporter Mdr49, a functional homolog of Ste6 – a geranylated germ cell attractant of *Drosophila melanogaster* has also been shown to exit cells (Ricardo and Lehmann, 2009). Moreover, proteins can be exported from parasites. Acylated proteins, such as hydrophilic acylated surface protein B (HASPB), are exported from various *Leishmania* species (Denny et al., 2000; Stegmayer et al., 2005; Maclean et al., 2012); an acylated protein kinase from *Plasmodium falciparum*, plasmodium falciparum Ca^{2+}-dependent protein kinase 1 (PfCDPK1), is exported to the parasitophorous vacuole (Möskes et al., 2004). These findings raise the possibility that, in a wide range of eukaryotic organisms, different kinds of lipidated peptides and proteins are externalized by specific ABC transporters. We classify these processes as type II unconventional secretion.

Type III – secretion through autophagosome-like vesicles

This type of unconventional secretion refers to cytoplasmic proteins, whose export involves intracellular vesicles as transport intermediates (Nickel and Rabouille, 2009). The secretion of interleukin 1β (IL1β) is one of the earliest examples of this form of secretion. (Rubartelli et al., 1990). Different types of vesicle, such as secretory lysosomes, microvesicles and multi-vesicular bodies, have been proposed to have a role in IL1β secretion (Andrei et al., 1999; MacKenzie et al., 2001; Andrei et al., 2004; Qu et al., 2007). However, the exact identity of vesicles that carry IL1β for secretion remains unclear. Secretion of IL1β is believed to depend on caspase-1-dependent proteolytic cleavage of a larger precursor, a process that then generates the biologically active, mature form of IL1β. However, although cleavage is induced by 'danger' signals that activate intracellular inflammasomes (Franchi et al., 2009; van de Veerdonk et al., 2011), substantial amounts of IL1β precursor were found in cellular supernatants – even in the absence of inflammasome activation (Keller et al., 2008). These findings suggest that precursor processing to the mature protein is not a strict requirement for IL1β secretion. It is possible that, in a constitutive manner, cells secrete IL1β precursor, which is then cleaved within the extracellular space into the mature and biologically active form of

IL1β upon activation of inflammasomes and secretion of activated caspase-1. Therefore, conclusions drawn from studies, in which solely the mature form of IL1β was analyzed in cellular supernatants (Keller et al., 2008), might be misleading because, primarily, activation of inflammasomes and caspase-1 rather than IL1β secretion was analysed.

The recent discovery that acyl-CoA-binding protein (AcbA) from *Dictyostelium* undergoes type III unconventional secretion (Kinseth et al., 2007; Cabral et al., 2010) might shed light on the uncertainty regarding the nature of these vesicular intermediates. Upon starvation, *Dictyostelium* cells secrete AcbA, which is cleaved in the extracellular space to generate a signaling peptide called SDF-2 (Anjard and Loomis, 2005). The yeast ortholog of AcbA is called Acb1 and is also secreted upon starvation (Duran et al., 2010; Manjithaya et al., 2010). Analysis of Acb1 secretion has revealed the involvement of a number of gene products including some of the proteins required for autophagy, leading to the suggestion that secretion of Acb1 involves an autophagosome-like vesicle (Duran et al., 2010; Manjithaya et al., 2010).

In addition to the involvement of factors that are required for the biogenesis of autophagosomes, such as phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P_3) and autophagy-related protein 9 (Atg9), the overall process of Acb1 secretion was shown to depend on a number of factors that are required for the fusion of endosomes, proteins of the multivesicular body pathway, such as the endosomal sorting complex required for transport 1 (ESCRT-I) component Vps23, the cell surface t-SNARE SSo1, as well as Grh1, the yeast ortholog of the mammalian peripheral Golgi reassembly-stacking proteins 1 and (GRASP65 and GRASP55, respectively; for a complete list see Poster) (Kinseth et al., 2007; Duran et al., 2010; Manjithaya et al., 2010; Vinke et al., 2011). Upon nutrient starvation, Grh1 concentrates in a PtdIns(3,4,5)P_3-kinase dependent manner to unique membranes near ER exit sites specific COPII component Sec13 (Bruns et al., 2011). These membranes are cup-shaped and are, therefore, named compartment for unconventional protein secretion (CUPS). CUPS contain PtdIns(3,4,5)P_3, Vps23 and the autophagy-related gene products Atg8 and Atg9.

The biogenesis of CUPS is starvation specific. Starvation also induces autophagy for the delivery of cellular components to the vacuole or lysosomes (Inoue and Klionsky, 2010; Yang and Klionsky, 2010). However, there are obvious differences between these two processes. Unlike autophagy, the biogenesis of CUPS is not triggered by rapamycin. Moreover, it involves Grh1, Bug1, and the ESCRT-II and III proteins Vps25, Vps36, Vps20 and Vps2 (Bruns et al., 2011), factors that are not required for classic autophagy. Interestingly, however, Vps34 is required for both the formation of CUPS and that of degradative autophagsomes. These findings highlight the involvement of a number of diverse proteins in the formation of CUPS and distinguishes this compartment from the starvation-induced formation of the degradative autophagy compartment preautophagosomal structure (PAS) and autophagosomes. On the basis of these findings, we propose that CUPS serve as a station for the biogenesis of autophagosomes that contain Acb1. These secretory autophagosomes do not fuse with the vacuole but, instead, fuse with the cell surface to release Acb1 into the extracellular space in an Sso1-dependent reaction (see Poster).

It has recently been reported that the autophagy-related gene product Atg5, the small GTPase Rab8a and GRASP55 (surprisingly, GRASP65 was not tested) are also required for unconventional secretion of IL1β (Dupont et al., 2011). It is, therefore, possible that secretion of a subset of unconventional secretory proteins such as IL1β is conserved and involves an autophagosome-like vesicular intermediate.

Type IV – Golgi bypass of proteins that reside in the plasma membrane

Besides the mechanisms described above, the term unconventional secretion has also been used to describe variations within the classic secretory pathway that is used for transport of membrane proteins to the plasma membrane (see Poster). Such mechanisms include the use of non-COPII-coated vesicular carriers during ER exit, such as for transport of the K^+ voltage-gated channel Kv4 (Hasdemir et al., 2005), as well as Golgi bypass of transmembrane proteins under defined physiological conditions. Golgi bypass is defined as the anterograde transport of transmembrane proteins from the ER to the plasma membrane without those proteins passing through the Golgi complex (reviewed by Grieve and Rabouille, 2011).

Golgi bypass is employed by an increasing subset of proteins that have the ability to reach the plasma membrane in a brefeldin A (BFA)-resistant manner (for a detailed summary of cellular effects of BFA, see Grieve and Rabouille, 2011). Briefly, BFA blocks ER-to-Golgi transport and, therefore, inhibits protein transport in the secretory pathway; thus, one of the criteria for Golgi bypass is BFA resistance. Although this requirement for Golgi bypass has not always been confirmed experimentally by inactivating key molecules, there are some examples that confirm this thought, such as the transport of the adhesion molecule integrin αPS1 in the *Drosophila* follicular epithelium at specific developmental stages (Schotman et al., 2008), and cystic fibrosis transmembrane conductance regulator (CFTR) (Yoo et al., 2002; Gee et al., 2011).

Indeed, CFTR, the best-characterized protein known to bypass the Golgi complex, can be transported to the plasma membrane in the absence of the Golgi t-SNARE syntaxin 5 or the small GTPases Sar1, ADP-ribosylation factor 1 (Arf1) and Rab1 (Yoo et al., 2002; Gee et al., 2011), which regulate coat formation and membrane trafficking in the early secretory pathway. Moreover, CFTR transport appears to require the t-SNARE syntaxin 13, which is specific for the late endosome (Yoo et al., 2002). The relevance of this finding may reside in the subsequent observation that CFTR transits through a specific intermediate compartment (IC) that is found in close vicinity to the centrosome (peri-centrosomal IC). From there it can reach recycling endosomes, thus allowing its transport to the plasma membrane (Marie et al., 2009; Prydz et al., 2012).

Golgi bypass is also used for the delivery of transmembrane proteins to specific subdomains of the plasma membrane, such as αPS1, which is delivered basolaterally to the *Drosophila* follicular epithelium (Schotman et al., 2008), or of certain pannexins (Penuela et al., 2007) and connexins to gap junctions (George et al., 1999; Qu et al., 2009). Other examples are the transport of serglycin, a multifunctional proteoglycan with role in immunity, hemostasis, cell growth and polarity (Scully et al., 2012) to the apical domain of MDCK cells (Tveit et al., 2009). Likewise, delivery of some proteins to cilia has been shown to bypass the Golgi complex (Hoffmeister et al.,

2011). Examples are polycystin 2, whose autosomal dominant and recessive mutation causes polycystic kidney disease (Chapin and Caplan, 2010) and Smoothened, a key component of the Hedgehog pathway (Corbit et al., 2005).

Not much is known with regard to the machinery that mediates a Golgi bypass (see Poster). The small GTPase Rab8 has been implicated in cilia formation and appears to be required to deliver polycystin 2 to cilia (Hoffmeister et al., 2011). GRASP protein family members (Vinke et al., 2011) were shown to participate to the mechanism that underlies the Golgi bypass of αPS1 in *Drosophila* (Schotman et al., 2008) and that of CFTR in HeLa cells (Gee et al., 2011). It seems that under conditions that prevent normal exit of CFTR from the ER, GRASP55 binds the last four C-terminal amino-acid residues of CFTR through its PDZ1 domain, thereby mediating its exit from the ER followed by transport to the plasma membrane in a Golgi-independent manner (Gee et al., 2011). Conditions that prevent ER exit are, for example, the expression of a misfolding CFTR mutant (ΔΦ508CFTR), or the inhibition of ER exit by overexpression of a dominant-negative form of Sar1 or Arf1 (George et al., 1999; Qu et al., 2009). Consistently, overexpression of either GRASP55 or GRASP65 rescues the viability and health of mice that carry the ΔΦ508CFTR mutation (Gee et al., 2011). Strikingly, as mentioned above, GRASP is also required for the type III unconventional secretion of both Acb1 (Kinseth et al., 2007) and IL1β (Dupont et al., 2011), where it marks sites on or near the ER from where secretory autophagosomes – the CUPS – form (Bruns et al., 2011; Deretic et al., 2012). Interestingly, ATG proteins also seem to have a role in the Golgi bypass of CFTR (Gee et al., 2011), thus outlining a second molecular resemblance in the mechanisms that underly type III and IV unconventional secretion. Whether GRASP family members and ATG proteins are also required for the Golgi bypass of other proteins remains to be investigated.

Conclusions and Perspectives

Not all proteins secreted by eukaryotic cells follow the conventional signal-sequence-dependent ER-Golgi pathway. On the basis of data available to date, the unconventional mode of protein secretion can be divided into four different types. As research interest in this area of biology is likely to grow with the number of identified unconventionally secreted proteins, we think it useful if the classification we have described here were adapted and followed. The identification of more unconventionally secreted proteins and their release mechanism from the cells will help in understanding how cellular proteins are secreted by eukaryotic cells depending on the signals and the cellular needs.

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