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Review

Pathways of Unconventional Protein Secretion

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Secretory proteins are conventionally transported through the endoplasmic reticulum to the Golgi and then to the plasma membrane where they are released into the extracellular space. However, numerous substrates also reach these destinations using unconventional pathways. Unconventional protein secretion (UPS) is complex and comprises cargos without a signal peptide or a transmembrane domain that can translocate across the plasma membrane, and cargos that reach the plasma membrane by bypassing the Golgi despite entering the endoplasmic reticulum (ER). With a few exceptions, unconventional secretion is largely triggered by stress. Here I review new results and concepts that are beginning to define these pathways.

From Classical Protein Secretion to UPS

The 50-year-old consensus states that secreted proteins and proteins integral to the plasma membrane reach their destination using the classical secretory pathway. Briefly, these proteins carry a **signal peptide** (see Glossary) and/or a transmembrane domain that direct their insertion into the ER from where they exit via **COPII-coated vesicles** to reach the Golgi apparatus and the plasma membrane [1] (for a recent review, see [2]). However, research over the past 15 years has shown that proteins can also be delivered to the plasma membrane and the extracellular space without entering the ER–Golgi conventional pathway of secretion [3,4]. This alternative route of secretion is called UPS [4].

Two categories of proteins are unconventionally secreted. The first comprises cytoplasmic 'leaderless' proteins that cross the plasma membrane and are active in the extracellular medium despite not having a signal peptide or a transmembrane domain. Note that these proteins may contain other signals that direct their secretion. Leaderless proteins are secreted along three pathways mediated by distinct mechanisms: Type I, or pore-mediated translocation across the plasma membrane; Type II, or ABC transporter-based secretion, which is dedicated to the secretion of acylated peptides and yeast mating peptides; and Type III or autophagosome/endosome-based secretion. The second category comprises proteins with a signal peptide and/or a transmembrane domain that enter the ER but bypass the Golgi apparatus on their way to the plasma membrane. This is the Type IV, or Golgi-bypass, pathway (Figure 1, Key Figure).

These pathways appear distinct but share common features. First, with a few notable exceptions they are induced by stress [5]. This is important because stress may cause impairment in the functional integrity of the classical secretory pathway, thus driving the need for efficient alternatives. Second, the leaderless substrates using the Type I and III pathways appear to directly translocate across membranes: the plasma membrane for Type I and the autophagosomal/endosomal membrane for Type III. Third, the Type III and IV pathways both use the peripheral Golgi proteins of the **Golgi Reassembly Stacking Protein (GRASP)** family [3].

Trends

With some notable exceptions, unconventional protein secretion (UPS) is largely triggered by cellular stress, inflammation, nutrient stress, endoplasmic reticulum (ER) stress, or mechanical stress.

Leaderless cytoplasmic proteins can be translocated across the plasma membrane through pores or via membrane-bound organelles. In the latter case, leaderless proteins are proposed to translocate across the membrane of the organelle.

Compartments for UPS (CUPSs) are not secretory autophagosomes and represent a mechanism for the concentration of Acb1 before it is secreted.

Point-mutant transmembrane proteins can bypass the Golgi on ER stress. ER stress triggers Golgi reassembly stacking protein 55 (GRASP55) phosphorylation and localization to the ER, where it mediates the Golgi bypass of certain cargos, whereas others depend on HSP70/DNAJ14.

Specific ciliary proteins also bypass the Golgi.

UPS has been observed in all organisms studied so far.

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Because the Type II pathway is not well studied, this review focuses on the mechanisms mediating the Type I, III, and IV pathways of unconventional secretion that are starting to emerge.

Type I Pathway: Plasma Membrane Pore Formation

The formation of plasma membrane pores allow cytoplasmic cargos known as cytoplasmic leaderless proteins to translocate across the plasma membrane. Regulated pore formation can be either self-sustained or driven by inflammation and is a key mechanism in the release of leaderless proteins from the cytoplasm to the extracellular space. The identification of proteins in different cell types as well as the pore they use to translocate is a key milestone to be reached in the future.

Self-Made Lipidic Pores Mediate Constitutive Secretion of Fibroblast Growth Factor 2 (FGF2) and HIV Transactivator of Transcription (TAT)

Although most unconventional secretion pathways are stimulated by stress, two proteins, **FGF2** [6,7] and HIV **TAT** [8,9] are constitutively secreted. These proteins form a self-made lipidic pore that allows them to translocate across the plasma membrane to reach the extracellular space (Figure 2). Their translocation comprises four steps. (i) Fully folded FGF2 [10] and HIV TAT [11] are recruited to the cytoplasmic leaflet of the plasma membrane by interaction with phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2), which is enriched there. FGF2 interaction with PI(4,5)P2 also drives its phosphorylation on Y82 by Tec kinase [12,13]. (ii) They undergo PI(4,5)P2-induced self-oligomerization at the plasma membrane [9]. (iii) Their oligomerization drives membrane insertion and pore formation. Based on *in vitro* experiments, oligomerized FGF2 and HIV TAT interacting with PI(4,5)P2 create a lipidic membrane pore with a toroidal architecture [9,14,15]. This pore allows their translocation to the extracellular membrane. (iv) Extracellular dissociated monomeric FGF2 [16] is trapped by heparan sulfate proteoglycans. It is, however, unclear whether the pore disassembles on one side of the cell while being formed on the other side or whether the pore is stable and allows translocation of monomeric FGF2 [7]. HIV TAT has been shown to bind to heparin sulfate [17] and it is tempting to speculate that this binding is necessary for its secretion as it is for FGF2.

In addition to PI(4,5)P2 and TEC kinase, ATP1A1, the alpha chain of the Na/K-ATPase, has recently been identified to play an important role in FGF2 secretion [18]. Its role appears to be independent of the ATPase complex. ATP1A1 has a strong affinity for FGF2 and is proposed to contribute to FGF2 recruitment to the cytoplasmic leaflet of the plasma membrane [18] (Figure 2). Whether ATP1A1 also participates in HIV TAT secretion remains to be established.

Why does constitutive secretion of FGF2 and HIV TAT not proceed through the classical secretory pathway? At least for FGF2, the Type I pathway may be used to avoid Golgi-based modifications and/or ensure quality control. When FGF2 was forced to use the secretory pathway by appending a signal peptide, it was secreted but not biologically active [19]. This is because it receives undesired modifications when passing through the Golgi, such as *O*-linked chondroitin. In addition, as FGF2 translocation is strictly dependent on its folding, it may constitute a quality control more stringent than the one applied in the ER, where the folding of its beta barrels might be suboptimal [10]. The same reasons may apply to HIV TAT but this is currently unknown.

Inflammation-Driven Heterologous Pore Formation

Translocation across the plasma membrane is also triggered by inflammation, which leads to massive and quick release of cytokines from the macrophage cytoplasm to the extracellular space.

Glossary

Acb1/A: small leaderless proteins secreted from yeast and *Dictyostelium* during the stress of glucose starvation. Once secreted they are cleaved to produce the small peptide SDF2, which acts as a potent sporulation factor.
Atg proteins: proteins encoded by the Atg genes (such as Atg5, Atg7, and Atg8/LC3). They regulate key steps of the autophagic process, a discovery that led to the award of the 2016 Nobel Prize in Physiology or Medicine to Japanese autophagy researcher Yoshinori Ohsumi.
Autophagy (macroautophagy): the orderly process by which cellular components are degraded and recycled. Targeted cytoplasmic constituents are isolated from the rest of the cell within double-membrane vesicle known as autophagosomes. They normally fuse with lysosomes to allow the degradation and recycling of their contents.
COPII-coated vesicle: a type of vesicle that transports proteins from the ER to the Golgi apparatus. It comprises six subunits and its formation is initiated by the recruitment of the small GTPase Sar1-GTP to the ER membrane.
Endoplasmic reticulum (ER) stress: series of cellular insults compromising ER homeostasis, such as accumulation of misfolded proteins, unbalance in redox activity, or decrease in calcium concentration. This elicits a series of responses including the unfolded protein response (UPR) that aims to restore ER function. If the damage to the ER is too severe and too protracted, the UPR aims toward apoptosis.
Endosomal sorting complex required for transport (ESCRT): machinery comprising cytosolic protein complexes known as ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III. Together with Vps4, the ESCRTs are able to remodel the membrane of late endosomes to allow the formation of small vesicles in the lumen of the endosomes.
Fibroblast growth factor 2 (FGF2): has broad mitogenic and angiogenic activities in the extracellular medium.
Golgi Reassembly Stacking Protein (GRASP): a family of myristoylated peripheral proteins localized to the Golgi apparatus that act as membrane tethers. In mammalian cells in steady state,

One of the most-studied cytokines is IL-1β. In response to inflammation, the leaderless protein IL-1β precursor is rapidly synthesized and cleaved by caspase 1 yielding mature IL-1β, which is secreted in an unconventional manner by a pathway that is a matter of intense debate [20] (see also Type III, below). IL-1β secretion from macrophages on inflammation appears to use the Type I pathway. However, IL-1β does not bind PIP2 and does not form pores. Instead, IL-1β release is concomitant with the hyperpermeabilization of the macrophage plasma membrane, which precedes cell lysis [21,22]. This hyperpermeabilization has been investigated using the drug punicalagin, which allows the uncoupling of IL-1β maturation from its secretion. On treatment with this drug, mature IL-1β accumulates but is not released, but on drug washout mature IL-1β is released within minutes from the cytosolic pool of intact macrophages [21].

What is the nature of this membrane hyperpermeabilization? Like the IL-1β precursor, the protein gasdermin is cleaved by caspase 1 on inflammation yielding the N-terminal half of the protein (gasdermin-N). This protein segment is efficiently recruited to the plasma membrane through binding PIP2, where it can form 16-fold-symmetry pores [23]. Although not formally proven, it is tempting to speculate that, at least in the early stages of inflammation, cytosolic mature IL-1β uses this pore for secretion. Inflammation would then lead to cell lysis and the release of other cytokines (Figure 2).

Another pore that forms on inflammation is the purinergic receptor P2X7 through which transglutaminase 2 (TG2) and thioredoxin, two leaderless cytoplasmic proteins, are also secreted by macrophages after stimulation that mimics inflammation [24] (Figure 2). However, these results raise several questions, as stimulation of P2X7 leads to the shedding of flottilin-positive microvesicles as well as IL-1β secretion. Furthermore, how P2X7 oligomerization is mediated is unknown.

It is not fully understood why these proteins do not use the conventional secretory pathway. Cytokine release occurs during the acute stress of inflammation, which may compromise the functional integrity of the classical secretory pathway. Furthermore, to cope with the quantity and speed needed to respond to inflammation, a large amount of IL-1β precursor would need to be stored in one of the compartments of the secretory pathway, with the risk of being detrimental for constitutive secretion. To circumvent this, the precursor could be translated from a stored mRNA pool, translocated to the ER, and transported, but this might not meet the demand for speedy release.

Cellular Stress and Pore Formation?

FGF1, a ubiquitously expressed proangiogenic protein of the same family as FGF2, is also a leaderless protein that is secreted but in response to stress [25]. Furthermore, unlike FGF2, FGF1 secretion requires the formation of a large multiprotein release complex comprising several other leaderless proteins (such as annexin A2, sphingosine kinase, synaptotagmin p40, and small calcium protein S100A3) that also appear to translocate [25] (Figure 2). However, many questions remain regarding their mechanism of action. Do they form a pore? Do they use a pore formed by other proteins that need to be identified? Why are they not secreted through the classical secretory pathway?

Type I Pathway: Similar to Bacterial Secretion?

Pore formation is also a mechanism used by bacteria for the secretion of proteins across a membrane [26]. Does pore formation in Type I UPS share features with a bacterial secretion mechanism? Bacteria have eight known secretion systems, most of which permit secretion across multiple membranes while three allow translocation across one membrane only. The bacterial secretion (Sec) system, which is structurally and mechanistically analogous to the

GRASP65 and 55 are required to maintain the integrity of the Golgi ribbon.

HIV transactivator of transcription (TAT): a small leaderless protein of 86–101 amino acids that is synthesized in the early steps of HIV infection. It functions as a transcriptional activator but is also secreted from infected cells, allowing neighboring cells to internalizing it and ensuring viral spread.

Signal peptide: a short peptide present at the N terminus of the majority of newly synthesized proteins that are destined for the classical secretory pathway. It mediates insertion into the ER.

Key Figure

Schematic Representation of Three Types of Unconventional Protein Secretion (UPS)

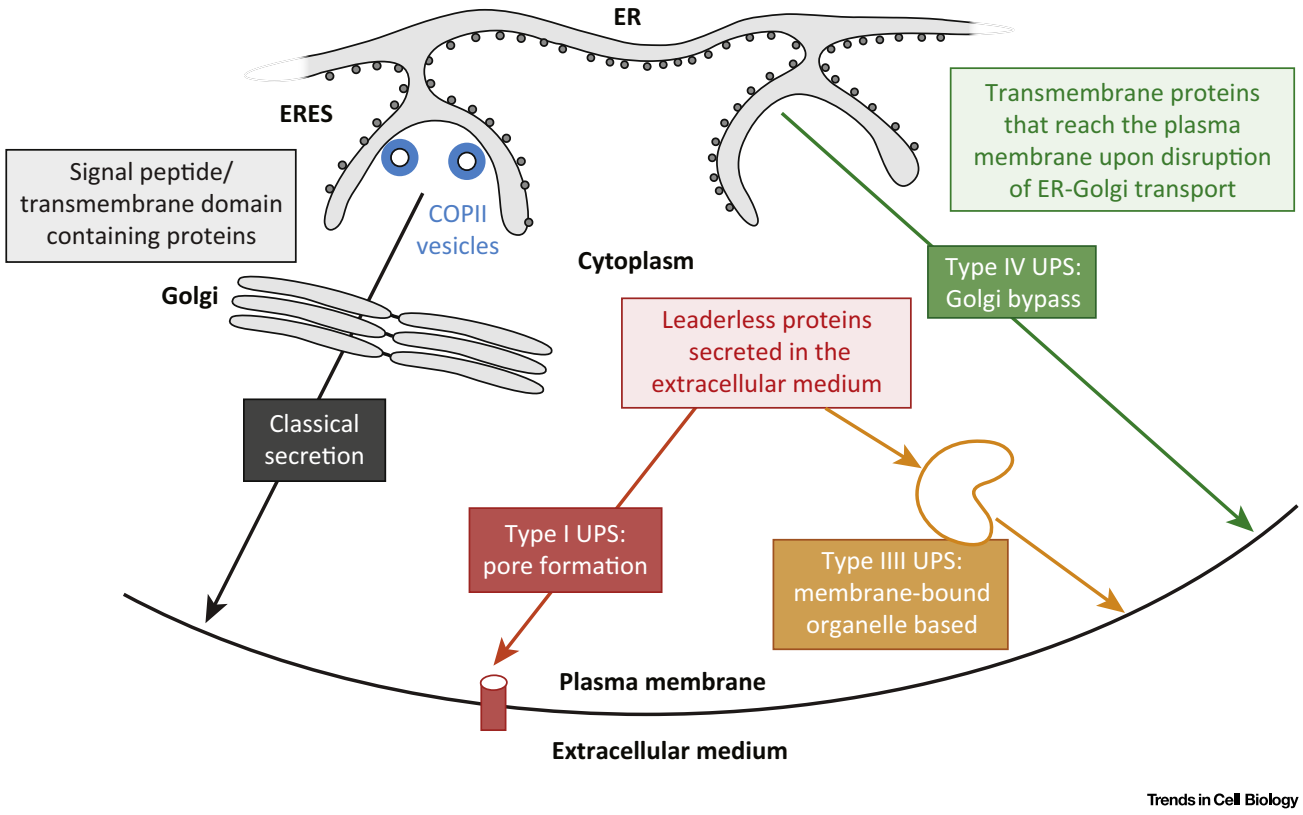

Figure 1. Leaderless proteins (red) can translocate across the plasma membrane through a pore (brown) or via a membrane-bound organelle (orange). Transmembrane proteins (green) can be transported to the plasma membrane using the classical secretory pathway (black) or the Golgi-bypass pathway (green).

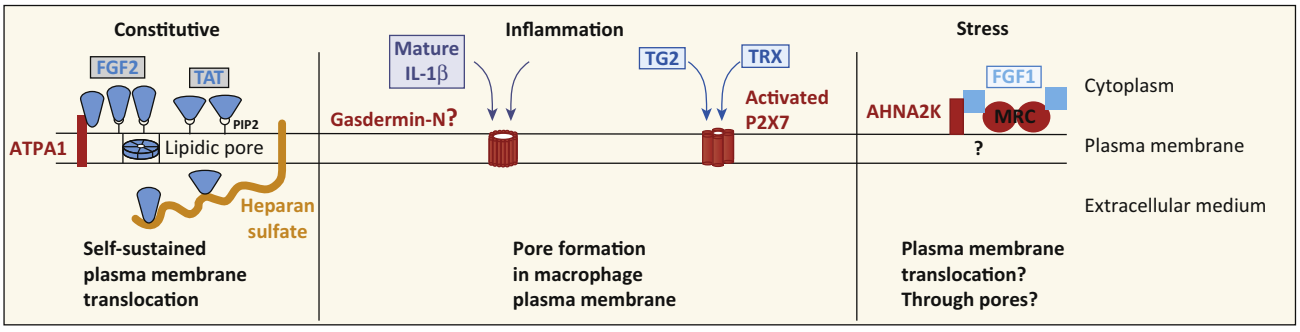

Figure 2. Pore Formation in Type I Unconventional Protein Secretion (UPS). Historically, Type I UPS has been defined by the constitutive, self-sustained, oligomerization-driven membrane insertion of fibroblast growth factor 2 (FGF2) and HIV transactivator of transcription (TAT). However, inflammation triggers IL-1β and transglutaminase 2 (TG2)/thioredoxin through heterologous pores that form at the macrophage plasma membrane. Furthermore, other leaderless proteins also translocate across the plasma membrane on stress using poorly defined mechanisms, such FGF1 and the membrane release complex (MRC).

conventional eukaryotic secretion translocon machinery, allows translocation of unfolded substrates while the Type I pathway translocates folded substrates only. In this respect, the twin-arginine translocase (Tat) system translocates folded substrates across the inner bacterial membrane. These substrates exhibit a Ser-Arg-Arg motif at their N terminus that is recognized by two of the Tat subunits and targeted to the channel formed by the third. The Tat system could therefore be a blueprint for heterologous pore formation and usage. However, the Tat system has not evolved in higher eukaryotes and, accordingly, the Ser-Arg-Arg motif is not present in, for instance, cytokine protein sequences. The Type V bacterial secretion system allows protein translocation from the periplasm across the outer membrane. Similar to FGF2 and HIV TAT, it is an autotransporter whereby proteins secrete themselves. They carry their own beta-barrel domains that insert into the outer membrane to form a channel that it used by the rest of the protein.

Type III Pathway: Organelle-Based Translocation of Leaderless Proteins

The stress-mediated Type III pathway relies on membrane-bound organelles that are diverted from their normal function and become secretory. These organelles are proposed to be endosomes and autophagosomes [27]. Late endosomes are already known to be 'secretory' and to release their internal vesicles (exosomes). However, several leaderless proteins do not utilize exosomes for their release [28–30]. Instead, they appear to translocate across the organelle membrane similarly to how they cross the plasma membrane in the Type I pathway.

From Atg Genes to Secretory Autophagosomes

The notion of secretory autophagosomes as a key step in the unconventional secretion of leaderless proteins has emerged during the study of yeast **Acb1** [28,31], a small leaderless protein secreted during stress on glucose starvation. As Acb1 secretion relies on Atg genes [28,31] and a plasma membrane SNARE, it raised the possibility that it is mediated by specialized secretory autophagosomes [4].

This discovery relaunched interest in **autophagy** as a mechanism regulating IL-1β secretion on stimulation by nutrient starvation. In addition to being a substrate of Type I UPS, IL-1β precursor has also been proposed to reach the lumen of a membrane organelle long thought to be an endo/lysosome, where it is converted into mature IL-1β. On fusion of this organelle with the plasma membrane, mature IL-1β is released into the extracellular space [32]. Several mechanisms have been proposed to account for IL-1β presence in an endolysosome [20], including its translocation across the lysosomal membrane using a transporter [32] and its capture by an autophagosome [33–36]. In this regard, IL-1β secretion is inhibited by the (albeit not very specific) autophagy inhibitors Wortmanin and 3′-methyladenine [36]. Furthermore, primary macrophages mutant for the key autophagy gene Atg5 [34], as well as Atg5-depleted reconstituted Hek293T cells [36], do not secrete IL-1β on starvation. Last, the autophagosome marker LC3 colocalizes with IL-1β [34,36]. Of note, the stimuli triggering IL-1β secretion appear to determine which secretory pathway is used. When inflammation is the trigger, IL-1β secretion utilizes the Type I pathway and appears quickly from a cytoplasmic pool, followed by cell death. When starvation is the trigger, IL-1β secretion goes through the Type III pathway via a membrane compartment and is not followed by cell death.

Although not induced by starvation, **Atg proteins** are also involved in the secretion of insulin degradation enzyme (IDE) by astrocytes [37]. This enzyme is critical for the efficient degradation of the APP peptide Aβ, the peptide that accumulates outside cells in Alzheimer disease. Importantly, IDE secretion is triggered by the presence of Aβ in the extracellular medium in a positive feedback loop. Under conditions where autophagy is impaired [for instance, in Atg7 knockout (KO) mice], Aβ does not trigger IDE release. This leads to Aβ extracellular accumulation and contributes to Alzheimer disease progression. Accordingly, deregulated autophagy is

reported to accelerate the progression of the disease [37]. It remains to be determined how extracellular Aβ triggers the autophagic flux and IDE release in healthy astrocytes.

While it is accepted that Atg proteins are involved in the unconventional secretion of several leaderless proteins, the role of secretory autophagosomes as mediators in this pathway is less clear. The first sign of caution comes from AcbA/1 secretion, which strictly depends on Grh1, a member of the GRASP family of peripheral Golgi proteins [4,28,38–40]. On glucose starvation Grh1 relocates from the ER–Golgi to one or two large cytoplasmic spots, the compartments for UPS (CUPSs). Importantly, CUPS formation (and Acb1 secretion) strictly depends on Grh1. Furthermore, IL-1β secretion on starvation also requires the two mammalian GRASP family members GRASP55 [34,36] and GRASP65 [36], with a role for GRASP55 in the formation of secretory autophagosomes [34]. It was therefore assumed that the CUPSs were secretory autophagosomes [41]. However, none of the Atg proteins appears necessary for CUPS formation in yeast [29,41,42]. Furthermore, besides Grh1, only a few factors are required for both Acb1 release and CUPS formation, suggesting that these two processes represent two sequential steps of Acb1 secretion. The first is the formation of mature CUPSs that contain Grh1. CUPSs are initially a collection of small vesicles and tubules that mature to an Acb1-positive, stable tubular compartment [29] in an **endosomal sorting complex required for transport (ESCRT)**-I-, II-, and III-dependent manner. Mature CUPSs are then encased in saccules (Figure 3), whose nature has not been clarified. The second step is the export of Acb1 from mature CUPSs to the exterior. While the exact mechanism remains unknown, one possibility is that Acb1 translocates into the flattened saccule (Figure 3), followed by the fusion of the saccule to the plasma membrane [29].

It is also becoming clear that Atg proteins have autophagy-independent functions [43]. Furthermore, caution should be exerted in the interpretation of LC3 colocalization with IL-1β, as it could

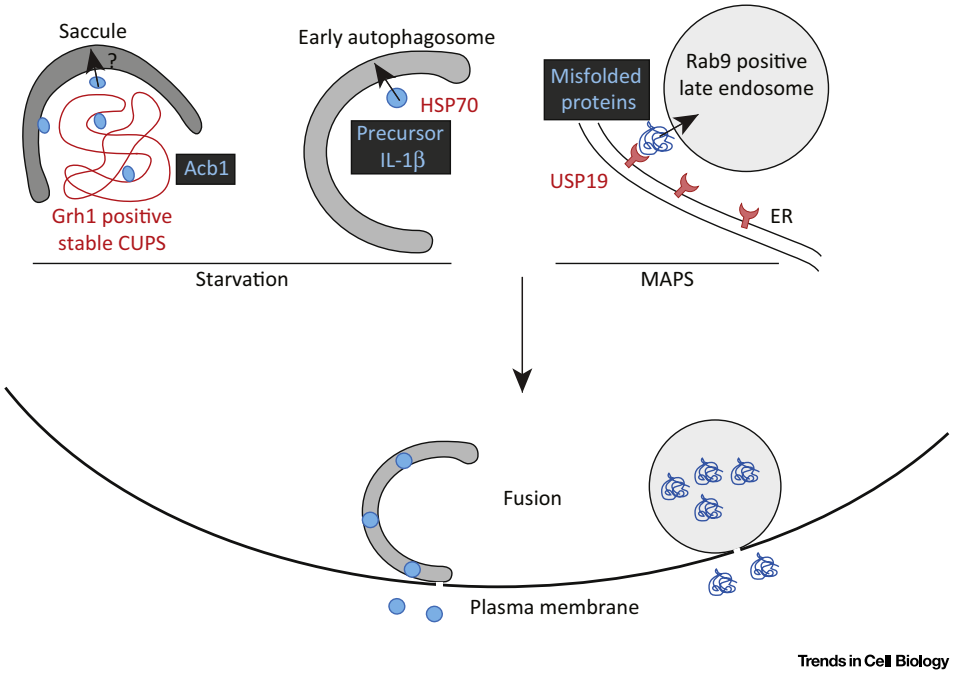

Figure 3. Translocation across Membrane Organelles in Type III Unconventional Protein Secretion (UPS). An emerging feature of Type III UPS is that substrates translocate across the membrane of the sequestering 'secretory' organelle: a saccule for Acb1 (proposed), an early autophagosome for IL-1β, and a late endosome for misfolded proteins (MAPS).

refer to degradation, not secretion. Last, the fusion of a secretory autophagosome with the plasma membrane remains to be demonstrated. Taking these findings together, the exact role of Atg proteins in the unconventional release of leaderless proteins remains to be clarified.

Protein Translocation across Organelle Membranes: Not Only for the Type I Pathway

An important issue with the extracellular release of leaderless proteins from secretory autophagosomes is that they are membrane bound (i.e., enclosed by the inner membrane of the autophagosome) and are therefore not soluble. Digestion of this membrane in the extracellular medium has been hypothesized but has remained speculative.

To circumvent this insolubility, proteins to be secreted could translocate across the inner membrane of the autophagosome, thus ensuring their release as a soluble protein on fusion. This has been shown for IL-1β *in vitro* [36] (Figure 3). Interestingly, a mechanism for protein translocation across the lysosomal membrane exists in the form of chaperone-mediated autophagy (CMA). Substrates exhibiting a KFERC motif are recognized by Hsp70 and brought onto the lysosome surface across which they are translocated in a LAMP2a-dependent mechanism before being degraded [44]. The motif necessary for IL-1β translocation has features resembling that used for CMA. However, IL-1β is not degraded but secreted, its translocation is proposed to occur across an early autophagosome membrane and not a lysosome, and it appears to depend on Hsp90 and not on Hsp70 as in CMA. The exact mechanism of this translocation remains to be established.

Membrane translocation of another membrane-bound organelle, the late endosome, has recently been proposed for the unconventional secretion of misfolded proteins that accumulate in the cytoplasm due to overloading of the proteasome [30]. The misfolding-associated protein secretion (MAPS) pathway describes how cellular stress-induced misfolded proteins are ubiquitinated, recognized by the ER-localized deubiquitylase USP19, and translocated into the lumen of an Rab9-positive endosome (Figure 3). These loaded endosomes are then proposed to fuse with the plasma membrane in a VAMP7/8-dependent manner, thus releasing misfolded proteins into the extracellular medium [30]. How this translocation is achieved is unclear, but given the presence of LAMP2a in late endosomes it could depend on a CMA-like pathway.

Type IV Pathway: Bypassing the Golgi with Signal Peptide/Transmembrane Domain-Containing Proteins

Despite also being triggered by cellular stresses such as ER and mechanical stress, the Type IV unconventional secretion pathway is distinct from the Type I and III pathways. It involves signal peptide- and/or transmembrane domain-containing proteins that are synthesized in the ER but bypass the Golgi when they are delivered to the plasma membrane. They reach the plasma membrane even in the presence of brefeldin A, a drug that disturbs ER-to-Golgi transport [45] and/or the absence of syntaxin 5. As a result of this bypass, these substrates harbor ER high-mannose oligosaccharides that are not processed by Golgi enzymes and cannot be cleaved by Golgi-localized proteases [45].

ER Stress Triggers Golgi Bypass via GRASP or Hsp70

Specific transmembrane proteins normally destined for the plasma membrane can bypass the Golgi by triggering **ER stress**. A point mutation in a transmembrane protein can prevent the full folding of that protein in the ER, which leads to its accumulation and the activation of ER stress pathways. For instance, protein C harboring the mutation A267 [46], which is associated with high risk of venous thrombosis, cannot reach the plasma membrane. The same is true for H723R-mutated pendrin, an anion transporter [47], and for Phe508-deleted cystic fibrosis transmembrane conductance regulator (CFRT), which leads to cystic fibrosis [48,49]. ER stress stimulates the exit of these mutated proteins from the ER and their delivery to the plasma

membrane but without passing through the Golgi. This relies on their recognition in the ER by sorting machineries, two of which have been identified.

The first sorting machinery is built around the GRASP family members. dGRASP was first identified in *Drosophila* for its role in the unconventional secretion of integrins [50] and GRASP55 was thereafter shown to be required for the Golgi bypass of mutant CFTR in mammals [49]. At steady state GRASP55 forms a homodimer that is localized at the Golgi. However, ER stress triggers the phosphorylation of serine 441 on GRASP55, which results in its monomerization and relocalization to the ER [51] (Figure 4). Tagging GRASP55 at the N terminus facilitates its ER localization, and this form has been used to rescue the cystic fibrosis phenotype in a mouse model [49]. At the ER monomeric GRASP55 recognizes the PDZ domain of specific 'Golgi-bypass' substrates (such as CFTR) via its own PDZ1 domain [40,49]. This creates a sorting mechanism followed by substrate encapsulation into carriers. It is unlikely that the carriers are COPII-coated vesicles as the expression of a dominant-negative form of Sar1 triggers Golgi bypass while preventing COPII vesicle formation. These carriers are then targeted directly to the plasma membrane (or possibly to endosomes [48]) without passing through the

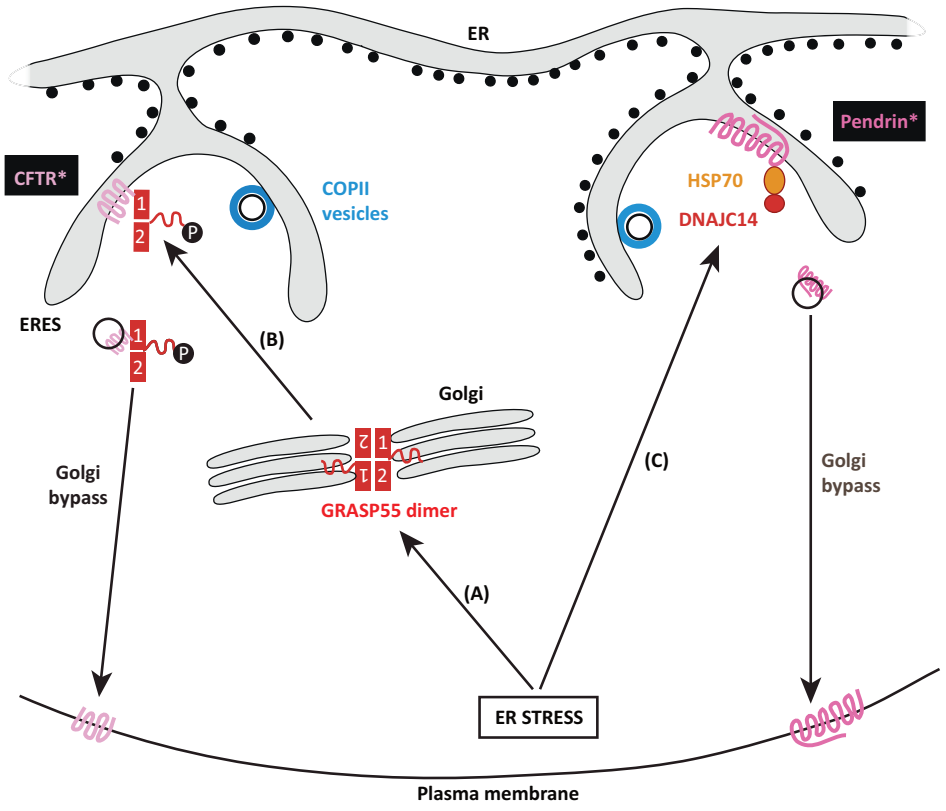

Figure 4. Endoplasmic Reticulum (ER) Stress-Mediated Golgi Bypass [Type IV Unconventional Protein Secretion (UPS)]. Two ER-retained mutated proteins, Phe508-depleted cystic fibrosis transmembrane conductance regulator (CFTR*) and S723T pendrin (pendrin*), are delivered to the plasma membrane without passing through the Golgi in an ER stress-dependent manner. ER stress induces the phosphorylation of Golgi reassembly stacking protein 55 (GRASP55) on serine 441, which leads to its monomerization (marked A). Golgi GRASP55 is represented as a dimer with its PDZ1 and 2 domains as red squares and its C terminus unstructured as according to [40]. Phosphorylated monomeric GRASP55 is relocalized to the ER and the ER exit site (ERES) where it interacts with CFTR* through its PDZ1 (marked B). ER stress also induces upregulation of DNAJC14, the co-chaperone of HSP70 (marked C), which binds pendrin*. This leads to CFTR* and pendrin* incorporation into carriers that bypass the Golgi and deliver them to the plasma membrane.

Golgi (Figure 4). The identification of the kinase required for GRASP55-specific phosphorylation and the mechanism of its ER-stress activation remain to be investigated.

The second sorting machinery is Hsp70 and its co-chaperone DNAJC14, which mediates Golgi bypass of H723R pendrin to the plasma membrane. Hsp70 interacts directly with H723R pendrin. Furthermore, ER stress leads to the upregulation of DNAJC14, which appears to retrieve the mutated protein from the ERAD pathway and to mediate its plasma membrane delivery (Figure 4) [47].

Questions remain to be answered regarding the role of ER stress in triggering Golgi bypass. What determines the use of the GRASP55-based machinery versus HSP70/DNAPC14? The presence of a PDZ domain at the C terminus of CFTR appears critical for the GRASP pathway, but is it the same for other transmembrane proteins? How are these carriers targeted to the plasma membrane without reaching the Golgi?

Mechanical Stress and Golgi Bypass

Mechanical stress has also been implicated in the Golgi bypass of integrins at a specific stage of *Drosophila* follicular epithelium development during oogenesis [5,50,52,53]. Interestingly, a subset of ciliary membrane proteins such as polycystin 2, M2-mutant Smoothened [54], and peripherin/rds [55] also bypass the Golgi and share features with integrin Golgi bypass. First, although not experimentally proven, mechanical stress (shear forces) produced by ciliary beating could induce the Golgi bypass of ciliary proteins. Second, integrins and cilia that bypass the Golgi are delivered to a small and defined region of the plasma membrane [50]. Third, both integrins and ciliary proteins are not mutated. Last, delivery of transmembrane proteins to other domains of the plasma membrane uses the classical secretory pathway, such as rhodopsin for the cilia [56], which suggests that the Golgi is functional and that specific signals elicit sorting to the Golgi-bypass pathway. However, dGRASP, which is required for integrin Golgi bypass in *Drosophila*, does not appear to be needed for the secretion of ciliary proteins. Are Hsp70 and DNAJC14 involved? If not, what is the machinery mediating ciliary protein Golgi bypass? Furthermore, it will be important to determine how mechanical stress triggers Golgi bypass and how it compares with ER stress. Does dGRASP also relocate to the ER? Are the carriers COPII-coated vesicles?

Similar to the Type I and III pathways, the Type IV pathway is clearly triggered by stress. However, how is Golgi-bypass cargo segregated from that using the classical secretory pathway? How does stress activate this specific sorting? Furthermore, why do certain transmembrane proteins (including point-mutant proteins) bypass the Golgi? Part of the answer lies perhaps in the resulting glycosylation profile. *N*-oligosaccharides forming on proteins are modified in the Golgi to become complex, whereas they remain high mannose when they bypass the Golgi. This difference in oligosaccharide processing may modify protein function at the plasma membrane in terms of oligomerization and binding affinity for ligands. This might be needed to respond to the cell's environment. Alternatively, the cargo segregation away from conventional cargos in the ER is perhaps a critical step; for instance, to avoid undesired complex formation in the ER and/or at the plasma membrane.

Concluding Remarks

Many questions remain to be answered (see Outstanding Questions), including whether unconventional secretion occurs in all eukaryotic organisms. So far all organisms examined, including yeast, *Dictyostelium*, *Drosophila*, and mammals [4], appear to have pathways for UPS. Unconventional secretion of leaderless proteins, such as endochitinase Cts1, also occurs in fungi (*Ustilago maydis*) [57]. In plants the only leaderless protein that appears to be unconventionally secreted is the merolectin Helja, a lectin from *Helianthus annuus* that is located at the apoplast (corresponding to the cell wall) of sunflower seeds [58,59]. However, the published

Outstanding Questions

How many proteins are secreted unconventionally? What proteins are secreted through a pore (Type I pathway), use a membrane-bound organelle (Type III pathway), or bypass the Golgi (Type IV pathway)?

Is this pool cell-type specific? Is this pool specific to a given stress?

How many of these proteins can form self-made pores?

How are leaderless proteins recognized? What is the signal in leaderless proteins resulting in their translocation through pores?

What is the biochemical composition of membrane-bound organelles that mediate secretion of leaderless proteins?

How does unconventional cargo translocate across the membrane of these organelles?

What is the role of Atg proteins in Type III unconventional secretion?

What mediates the fusion of these organelles with the plasma membrane?

What type of carriers do signal peptide/transmembrane domain-containing proteins use to exit the ER and be delivered to the plasma membrane while bypassing the Golgi?

How does ER and mechanical stress trigger Golgi bypass?

How do GRASP family members function in Type III and IV UPS?

Arabidopsis secretome includes several leaderless proteins that appear to localize to the cell wall or the apoplastic proteome [60–63]. The value of proteomics data is dependent on the purity of the organelle or subcellular fraction, but combined these results suggest that UPS of leaderless proteins exists in plants. Of note, this is GRASP independent as GRASP does not have an ortholog in plants.

Conversely, examples of fungal BFA-insensitive secretion of signal peptide-containing proteins are the chitin synthases of *Neurospora crassa* and a cellobiase of the fungus *Termitomyces clypeatus* [57]. Furthermore, parasite fungi secrete small effector proteins in the cytoplasm of the host cell to manipulate it and facilitate colonization. This is mediated by an invasive hypha that induces a large invagination or inclusion of the host-cell plasma membrane. Most of these effectors have a signal peptide and some are secreted to the tip of the hypha using the classical secretory pathway. However, some of the effectors reach either the invasive hypha plasma membrane or the cytoplasm of the host cell in a structure called BIC in a BFA-resistant manner and are thus likely to bypass the Golgi. This Golgi bypass seems to depend on Sec5 and Sso1 [64]. Interestingly, Golgi bypass has so far been restricted to transmembrane proteins, not secreted ones, which opens the scope of this pathway.

The field of UPS is diverse and heterogeneous, uncovering many pathways to reach the plasma membrane and extracellular medium. This diversity is surely dictated by the nature of the cargos that are released, the conditions triggering their release (stress), and the cell type, but this is not clearly understood. Despite this diversity similarities start to emerge, like their triggering by cellular stress, the use of the GRASP family members, and the existence of pores to translocate across membranes. To gain a more unified and precise picture of the UPS field will require identification of the substrates using these pathways. This will then allow the deciphering of signals on the released cargos. Furthermore, the machineries sustaining the three UPS pathways need to be identified, as well as their regulation by stress. Only then will we gain a better understanding of why such alternative routes exist.

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