Primer

Unconventional mechanisms of eukaryotic protein secretion

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A big surprise in the molecular cell biology of eukaryotes has been the discovery of pathways of protein secretion that are not linked to the endoplasmic reticulum (ER) and the Golgi apparatus. Various kinds of unconventional secretory processes have been described, including two major pathways for two distinct sets of cargoes that are initially synthesized as soluble proteins in the cytoplasm. These two pathways are mechanistically distinct from one another. One is based upon direct protein translocation across lipidic pores in the plasma membrane (type I unconventional secretion). The second pathway involves the recruitment of cytoplasmic proteins into vesicular compartments of the endocytic membrane system that fuse with the plasma membrane to release proteins into the extracellular space (type III unconventional secretion). This primer highlights the mechanisms and molecular machineries of these pathways that were discovered with fibroblast growth factor 2 (FGF2; type I) and acyl-CoA binding protein (Acb1; type III) as the most prominent cargo proteins. Furthermore, the physiological significance of these secretory routes in both health and disease is discussed for a broader range of cargo proteins.

Diversity of secretory mechanisms in eukaryotic cells

For decades following the discovery of the ER–Golgi-dependent secretory pathway in eukaryotic cells (Figure 1A), it has been believed that all proteins destined for the extracellular space travel along this route. However, in the late 1980s, the first reports appeared proposing alternative mechanisms of eukaryotic protein secretion to exist along with a number of possible secretory cargo proteins. The underlying molecular mechanisms only slowly emerged, resulting in the identification of four distinct classes of unconventional secretory mechanism in eukaryotic cells (Figure 1). Three of these concern soluble proteins in the cytoplasm that are either secreted through direct protein translocation across lipidic pores in the plasma membrane (type I; Figure 1B), plasma-membrane-resident ABC transporters with the cargo proteins being modified by acylation (type II; Figure 1C), or uptake into endocytic compartments that mature and subsequently fuse with the plasma membrane (type III; Figure 1D). A fourth variant of unconventional secretion refers to plasma-membrane-resident integral membrane proteins that are inserted into the ER, but bypass the Golgi while traveling to the cell surface (type IV; Figure 1E). In this primer, we focus on the type I and type III pathways of unconventional secretion where recent studies have elucidated the molecular mechanisms involved in the greatest detail.

Type I unconventional secretion of FGF2 from tumor cells

FGF2 was among the earliest examples of unconventionally secreted proteins that were discovered in the late 1980s. This case was particularly compelling since the extracellular localization of this signal-peptide-lacking protein was linked to a well-established function — the formation of a ternary signaling complex of FGF2 with heparan sulfates and FGF high-affinity receptors on the cell surface of target cells. In this way, extracellular FGF2 transmits signals into cells that regulate a wide range of physiological responses, such as cell proliferation and differentiation. Furthermore, FGF2 signaling is not only critical during development but also plays prominent roles under pathophysiological conditions, promoting survival through an autocrine secretion/signaling pathway that protects tumor cells from programmed cell death.

Following a long period of very limited insight into the molecular mechanism by which tumor cells secrete FGF2, the emergence of advanced techniques, such as functional genomics based on RNAi screening, as well as the development of sophisticated membrane reconstitution systems allowed for a breakthrough. An important finding was the discovery that FGF2 contains a highly specific binding pocket for the head group of phosphatidylinositol (4,5) bisphosphate ($PI(4,5)P_2$), a membrane lipid enriched at the inner leaflet of the plasma membrane. Studies from our lab in 2012 showed that this interaction triggered oligomerization of FGF2 followed by membrane insertion and the formation of a lipidic membrane pore with a toroidal architecture. Another key result was the discovery that membrane-proximal heparan sulfates at the outer leaflet are not only required for FGF2 signaling but also essential for FGF2 secretion into the extracellular space. These findings were corroborated by experiments demonstrating that FGF2 variant forms that are defective in binding to either $PI(4,5)P_2$ or heparan sulfates fail to get secreted from tumor cells. Biochemical and structural analyses revealed overlapping binding sites for $PI(4,5)P_2$ and heparan sulfates on the molecular surface of FGF2, resulting in mutually exclusive interactions of FGF2 with these two binding partners.

These studies triggered the assembly/disassembly hypothesis of FGF2 membrane translocation, which proposed that membrane-inserted FGF2 oligomers act as dynamic translocation intermediates that are assembled at the inner leaflet in a $PI(4,5)P_2$-dependent manner (Figure 1B). Based upon high-affinity interactions of membrane-proximal heparan sulfates (K_D = 10 nM) that outcompete interactions of $PI(4,5)P_2$ with FGF2 (K_D = 1 μM) at the outer leaflet, membrane-inserted oligomers become disassembled, resulting in translocation of FGF2 to the cell surface. This model provides a mechanism that explains the directional transport of FGF2 from the cytoplasm into the extracellular space and is consistent with the observation that membrane translocation requires FGF2 to be properly folded during all stages of this process.

Recent work from our lab provided support for this hypothesis through a full reconstitution of the core mechanism of FGF2 membrane translocation using purified components (Steringer *et al.* (2017)). Using an inside-out membrane model

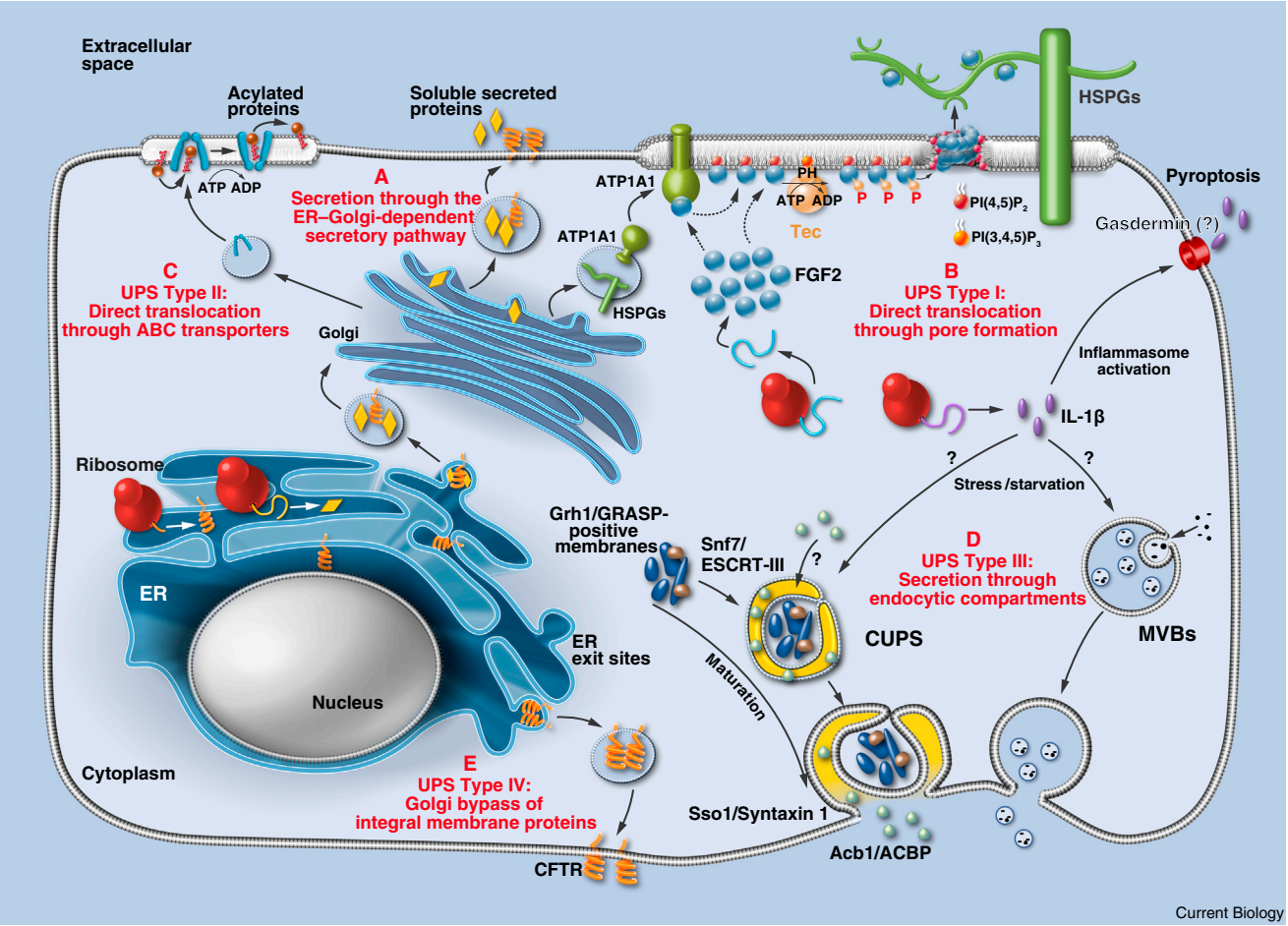

Figure 1. Schematic representation of secretory pathways in eukaryotic cells.
Beyond the classical, ER–Golgi-dependent secretory pathway (A), four types of unconventional protein secretion (UPS) have been described. Those include direct protein translocation across lipidic pores in the plasma membrane (B; UPS type I), secretion through plasma-membrane-resident ABC transporters with the cargo proteins being modified by acylation (C; UPS type II), secretion through uptake into endocytic compartments that mature and subsequently fuse with the plasma membrane (D; UPS type III) and transport of integral membrane proteins towards the plasma membrane bypassing the Golgi (E; UPS type IV). For details, see text.

system based upon giant unilamellar vesicles (GUVs), it was shown that $PI(4,5)P_2$ and heparan sulfates reconstituted on opposing sides of the membrane along with the ability of FGF2 to oligomerize and to form membrane pores in a $PI(4,5)P_2$-dependent manner are together sufficient as *trans* elements to drive FGF2 membrane translocation. This process depends on three critical *cis* elements in FGF2: the residues that form the binding pocket for $PI(4,5)P_2$; the residues forming the binding surface for heparan sulfates; and a cysteine residue in position 95 (Cys95) on the molecular surface of FGF2 that is critical for $PI(4,5)P_2$-dependent oligomerization. Cys95 forms intermolecular disulfide bridges that are required for the biogenesis of membrane-inserted oligomers with 8 to 12 subunits being the threshold for the formation of membrane pores. All three of these *cis* elements have been confirmed to be required for FGF2 secretion from cells. In conclusion, as a result of the competing interactions of FGF2 with $PI(4,5)P_2$ versus heparan sulfates on opposing sides of the plasma membrane, the core process of unconventional secretion of FGF2 from cells is based upon an unusual mechanism of self-sustained membrane translocation with the cargo protein forming its own membrane translocation intermediates by reversible oligomerization, membrane insertion and disassembly in the extracellular space.

In a cellular context, beyond the core mechanism of FGF2 membrane translocation established through biochemical reconstitution experiments, additional auxiliary components have been identified that support unconventional secretion of FGF2 from cells. Genome-wide RNAi screening identified two additional *trans*-acting factors,Tec kinase and ATP1A1. Both factors are physically associated with the plasma membrane, Tec kinase through a pleckstrin homology (PH) domain that binds to $PI(3,4,5)P_3$, and ATP1A1 as the α-subunit of the Na^+/K^+ ATPase. Direct interactions of FGF2 with both Tec kinase and the cytoplasmic domain of ATP1A1 have been demonstrated with sub-micromolar affinities. While FGF2

membrane translocation reconstituted with purified components does not depend on Tec kinase and ATP1A1, FGF2 secretion from cells is impaired when either Tec kinase or ATP1A1 are downregulated by RNAi. The cytoplasmic domain of ATP1A1 might play a role as the initial contact site at the plasma membrane, facilitating the accumulation of FGF2 at the inner leaflet. By contrast, Tec kinase has been shown to phosphorylate FGF2 at tyrosine 81 (Tyr81), a modification that appears to increase the membrane insertion efficiency of FGF2 oligomers that serve as translocation intermediates. Since Tyr81 is located in the direct spatial vicinity of Cys95, it has been proposed that tyrosine phosphorylation of FGF2 tunes the formation of disulfide bridges required for FGF2 oligomerization and membrane insertion. Therefore, auxiliary factors such as ATP1A1 and Tec kinase might be organized together with the core components $PI(4,5)P_2$ and heparan sulfates within microdomains in the plasma membrane that represent structural units coordinating FGF2 membrane translocation in space and time in living cells.

Type III unconventional secretion of AcbA/Acb1 from lower eukaryotes

The type III pathway of unconventional secretion was originally discovered in 2007 when Malhotra and colleagues turned to *Dictyostelium* as a model system to study the role of GRASP proteins (termed grpA in *Dictyostelium*) in Golgi biogenesis. This was because *Dictyostelium* cells express only one form of GRASP, whereas yeast and mammalian cells express two, hampering clear conclusions about their individual roles in the post-mitotic re-emergence of Golgi stacks. Surprisingly, upon starvation, the main phenotype of a *Dictyostelium* GRASP knock-out strain was a failure to form viable spores. It turned out that this process failed in the absence of GRASP because a signaling peptide (SDF-2) required for the induction of sporulation was missing. SDF-2 is synthesized as part of a precursor protein, acyl-CoA-binding protein (AcbA in *Dictyostelium*, Acb1 in yeast and ACBP in mammalian cells). Intriguingly, when *grpA*$^-$ cells were incubated with recombinant AcbA, sporulation resumed because the protease that mobilizes SDF-2 from the AcbA precursor protein is a plasma-membrane-resident enzyme with its active center facing the extracellular space. Since AcbA must get transported into the extracellular space for cleavage to produce SDF-2 and lacks a signal peptide, it was proposed to follow an unconventional route of secretion.

Intriguingly, Acb1, the yeast ortholog of AcbA, was also found to be secreted from yeast cells upon starvation, making the analysis of unconventional secretion of AcbA/Acb1 amenable to genetic screening procedures to identify factors involved in this pathway. It was found that Acb1 secretion in yeast cells also depends on the yeast GRASP ortholog Grh1 and led to the discovery of Acb1 secretion being dependent on a set of gene products required for autophagy as well as on the plasma membrane SNARE protein Sso1. At the same time, Subramani and colleagues published similar results from a different organism, *Pichia pastoris*. These findings were initially interpreted in terms of a direct role for autophagosomes in capturing Acb1 in the cytoplasm to follow a pathway of autophagy that does not end up with degradation of cargo proteins in lysosomes. Instead, it was proposed that this population of autophagosomes fuses with recycling compartments of the endocytic system. Upon fusion of such vesicular intermediates with the plasma membrane in an Sso1-dependent manner, Acb1 would be released into the extracellular space.

In follow-up studies using starvation as a trigger, it was shown that an early event in the Acb1 secretion pathway is the formation of a cup-shaped compartment whose biogenesis depends on Grh1. It was termed CUPS for Compartment of Unconventional Protein Secretion for the type III pathway and proposed to mediate the initial sorting of Acb1 into its unconventional secretory pathway. This compartment is located in the vicinity of ER exit sites, contains Grh1 and the ESCRT component Vps23 but lacks factors derived from the Golgi and endosomes. As discussed above, initial results suggested that the CUPS structure represents a subpopulation of autophagosomes since colocalization with autophagy-related proteins was observed. However, later studies challenged these findings, suggesting that CUPS is not directly related to autophagosomes. Recent work showed that starvation causes maturation of CUPS from immature forms into a stable structure that contains Acb1 and is characterized by a membrane-bound saccule with a heterogeneous population of vesicles and tubules sequestered inside (Figure 1D). The formation of this structure depends on the ESCRT complexes I, II and III, but is independent of Vps4, an AAA-ATPase that cooperates with ESCRT complexes in the formation of multivesicular bodies. Instead, another factor, Snf7, is recruited to the maturing CUPS compartment and is important for the biogenesis and the structural integrity of the saccule (Curwin *et al.* (2016)). Subsequent processes involve Sso1-dependent fusion of CUPS with the plasma membrane, resulting in Acb1 secretion into the extracellular space. While the details of the maturation of the CUPS structure and late steps in CUPS-mediated secretion of Acb1 remain to be resolved, these studies provide a fundamental explanation of how autophagosomes and MVBs differ both structurally and functionally from the CUPS compartment that is the central mediator of unconventional secretion of Acb1 as part of the type III pathway (Figure 1D).

Range of cargo proteins secreted by type I and type III pathways

The broad significance of type I and type III pathways for other secretory proteins that bypass the ER–Golgi system is only beginning to emerge. A comprehensive review of our current knowledge about the range of cargo proteins for all four types of unconventional secretion has been published recently (Rabouille (2017)). Here, we highlight selected examples for type I and type III secretion (Table 1). Beyond FGF2, the most compelling example described so far for another cargo protein secreted through a type I mechanism is HIV-Tat, which is secreted from HIV-infected T cells through an unconventional pathway

that depends on $PI(4,5)P_2$. Later, it was demonstrated that HIV-Tat shares many features of FGF2, including the ability to form oligomers and membrane pores in a $PI(4,5)P_2$-dependent manner. Recently, a direct role for ATP1A1 in the unconventional secretion of HIV-Tat has been reported, another striking similarity with FGF2. Interestingly, under experimental conditions that trigger an inflammatory response from macrophages, interleukin-1β (IL-1β) secretion also appears to follow a type I pathway that is linked to a process called pyroptosis. While IL-1β itself does not have the ability to directly bind to $PI(4,5)P_2$ to form membrane pores, it has been suggested that, in this case, the observed pores might be formed by another factor, through which IL-1β may reach the extracellular space. Indeed, a protein termed Gasdermin is activated by proteolytic cleavage upon inflammasome activation, with its active amino-terminal fragment driving membrane pore formation in a $PI(4,5)P_2$-dependent manner. Additional candidates that follow a type I mechanism of unconventional secretion are annexin A2 and members of the galectin family of proteins (Table 1).

Intriguingly, IL-1β is also capable of entering the type III pathway in macrophages, depending on a combination of stimuli that trigger autophagy and inflammasome activation (Figure 1D). This process has also been studied by Schekman and colleagues in non-macrophage cell lines, defining specific conditions that lead to the capture and unconventional secretion of mature IL-1β by the type III pathway. It is, however, unclear whether the vesicular intermediates involved in this process are directly related to the CUPS compartment described for Acb1 secretion from yeast cells (as discussed in the previous section). Instead, both the Deretic and the Schekman laboratories related these vesicular carriers to autophagosomes and termed the overall process 'secretory autophagy' in mammalian cells. It therefore remains to be established whether Acb1 secretion from yeast cells and IL-1β secretion triggered by starvation of mammalian cells are directly related in terms of molecular machinery and mechanism. In any case, biochemical reconstitution experiments provided direct evidence that IL-1β does not reach the most inner lumen of autophagosomal membranes (corresponding topologically to the cytoplasm) but rather translocates into the intermembrane space of these membrane-bound compartments (topologically equivalent to the extracellular space). Upon fusion of these vesicular intermediates with the plasma membrane, this localization would allow for the direct release of IL-1β into the extracellular space (Figure 1D). The processes described for IL-1β all depend on GRASP directly linking these pathways to what has been originally found for AcbA/Acb1 in *Dictyostelium* and yeast cells (see above). In addition, all of these processes depend on several gene products required for the induction of autophagy, factors involved in the biogenesis of MVBs, and dedicated SNARE proteins that mediate fusion events at the plasma membrane, separating this process from membrane trafficking pathways delivering proteins to lysosomes for degradation. The latter is strongly supported by experiments demonstrating that secretory autophagy is unaffected or, in fact, even enhanced when membrane fusion of autophagosomes with lysosomes is blocked. Thus, under conditions that trigger autophagy, such as starvation, AcbA/Acb1 and IL-1β are cargo proteins that define the type III pathway of unconventional secretion in various organisms (Table 1). Recent studies have shown that superoxide dismutase 1 (SOD1), which is linked to amyotrophic lateral sclerosis (ALS), is secreted by the type III pathway. Intriguingly, sorting of SOD1 into the

Table 1. Examples of cargo proteins following an unconventional secretory pathway.

UPS type	Cargo proteins	Organism	Extracellular functions
I	FGF2	*H. sapiens*/ *C. griseus*	Pro-angiogenic factor
	HIV-Tat	*H. sapiens*	Immune suppression, viral spread
	FGF1	*M. musculus*	Pro-angiogenic factor
	IL-1β*	*H. sapiens (macrophages)*	Inflammatory functions
	Annexin A2*	*H. sapiens*	Anti-angiogenic functions
	Galectin-1*	*C. griseus*	Regulator of proliferation and differentiation
	Tau*	*H. sapiens*	Propagation of Alzheimer's disease
	TG2*	*H. sapiens*	Cell adhesion, pro-inflammatory functions
II	MATa	*S. cerevisiae*	Mating
	Germ cell attractant	*D. melanogaster*	Cell migration
	HASPB*	*L. major*	Parasite virulence
III	AcbA	*D. discoideum*	Sporulation
	Acb1	*S. cerevisiae*	Sporulation
	IL-1β*	*H. sapiens*	Inflammatory functions
	SOD1	*S. cerevisiae*	Propagation of ALS disease
	Tau*	*H. sapiens*	Propagation of Alzheimer's disease
	TG2*	*H. sapiens*/ *M. musculus*	Cell adhesion, pro-inflammatory functions
IV	(ΔF508) CFTR	*H. sapiens*	cAMP-activated anion channel/cystic fibrosis
	aPS1 integrin	*D. melanogaster*	Cell adhesion

*Proteins without a well-defined/studied unconventional protein secretion (UPS) pathway. FGF1, FGF2, fibroblast growth factor 1 or 2; IL-1β, interleukin-1β; TG2, transglutaminase 2; HASPB, hydrophilic acylated surface protein B; SOD1, superoxide dismutase 1; CFTR, cystic fibrosis transmembrane conductance regulator.

type III pathway depends on a diacidic motif that is also present in AcbA/Acb1.

Physiological significance of unconventional secretory pathways

Why do eukaryotic cells maintain unconventional pathways of protein secretion? One reason might be that specific cargo proteins, such as lectins, are incapable of traveling within the lumen of the ER–Golgi system due to premature binding to their glycosylated counter receptors. In addition, it has been shown that adding an artificial signal peptide to FGF2 allows for secretion via the ER–Golgi pathway, but that under these conditions FGF2 becomes O-glycosylated, rendering the secreted protein biologically inactive. Another aspect of note is that, as discussed above, many unconventional secretory processes are triggered by stress conditions that may limit the function of the ER–Golgi system. Therefore, a specific set of cargo proteins may depend on alternative pathways that are fully functional under such circumstances. Also, conditions of stress might harm other cellular functions, such as protein degradation mediated by proteasomes. Recent work has identified a type III pathway of unconventional secretion that clears cells of misfolded proteins under conditions that do not allow for degradation of damaged proteins. Finally, unconventional secretory pathways allow for the secretion of proteins with biological functions both inside and outside cells. Therefore, depending on the physiological context, these mechanisms permit the dynamic distribution of proteins between the extracellular space and the cell interior, as is the case for FGF2 with functions both on cell surfaces and in the nucleus.

Future directions

Detailed insight regarding the secretory mechanisms of FGF2 and AcbA/Acb1 has been obtained from tumor cells and lower eukaryotes, respectively, defining the type I and III pathways of unconventional protein secretion. However, as discussed above, for many extracellular proteins exported from cells by unconventional means, their pathway of secretion and the molecular mechanisms involved remain to be identified (Table 1). Depending on the physiological context, it might even turn out that certain cargo proteins, such as IL-1β, can enter multiple pathways of unconventional secretion. Likewise, many questions remain regarding the cargo proteins and the mechanisms that mediate type II and type IV unconventional secretion. While specific examples of lipidated cargo proteins and peptides have been demonstrated to get exported from eukaryotes by plasma-membrane-resident ABC transporters, it is currently unclear whether these are rare exceptions or the first examples of a broad phenomenon. Likewise, it is currently unclear whether known cargo proteins of the type IV pathway, such as CFTR (Table 1 and Figure 1E), are rare and unusual cases of protein transport along the ER–Golgi route or whether a given physiological context such as cellular stress might have a broad impact on the classical secretory pathway, rerouting many proteins to ensure proper protein targeting under these conditions. In any case, it is fascinating to observe that eukaryotic cells evolved a broad variety of secretory mechanisms that are much more complex than previously assumed. It will be a challenge to reveal both the molecular mechanisms and the physiological significance of these pathways for a larger set of cargo proteins as well as to make use of this knowledge to develop highly specific drugs that prevent the secretion of individual proteins under pathophysiological conditions.

DECLARATION OF INTERESTS

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