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Review

Unconventional secretion mediated by direct protein self-translocation across the plasma membranes of mammalian cells

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In recent years, a surprisingly complex picture emerged about endoplasmic reticulum (ER)/Golgi-independent secretory pathways, and several routes have been discovered that differ with regard to their molecular mechanisms and machineries. Fibroblast growth factor 2 (FGF2) is secreted by a pathway of unconventional protein secretion (UPS) that is based on direct self-translocation across the plasma membrane. Building on previous research, a component of this process has been identified to be glypican-1 (GPC1), a GPI-anchored heparan sulfate proteoglycan located on cell surfaces. These findings not only shed light on the molecular mechanism underlying this process but also reveal an intimate relationship between FGF2 and GPC1 that might be of critical relevance for the prominent roles they both have in tumor progression and metastasis.

Highlights

Unconventional protein secretion (UPS) is a term collectively used for different kinds of endoplasmic reticulum (ER)/Golgi-independent secretory pathways that have been classified into subtypes that are either mediated by direct protein translocation across the plasma membrane (type I and II UPS) or involve vesicular transport intermediates inside cells (type III and IV UPS).

A unifying principle of type I UPS cargo proteins such as fibroblast growth factor 2 (FGF2), HIV-Tat, Tau, and EN2 is their ability to directly bind to $PI(4,5)P_2$.

FGF2 is a type I UPS cargo protein that can self-translocate across the plasma membrane, a process that depends on $PI(4,5)P_2$-dependent oligomerization at the inner leaflet and heparan sulfates at the outer plasma membrane leaflet.

In intact cells, the Na,K-ATPase plays a key role in unconventional secretion of FGF2 being required for the initial recruitment of FGF2 at the inner plasma membrane leaflet.

Glypican-1 (GPC1) is a GPI-anchored heparan sulfate proteoglycan that drives unconventional secretion of FGF2 with FGF2 capturing at the outer leaflet being the rate-limiting step of this process.

The intimate relationship between GPC1 and FGF2 as part of its type I UPS pathway has important implications for their roles in tumor progression and metastasis.

Pathways of unconventional protein secretion

The majority of secretory proteins are exported from cells based upon the ER/Golgi-dependent pathway [1–4]. However, a distinct set of extracellular proteins with fundamental functions in cell survival and migration, angiogenesis, and inflammation, as well as development under normal and pathophysiological conditions, are known to be secreted by alternative mechanisms. While the phenomenon of secretory proteins lacking signal peptides for ER/Golgi-mediated protein secretion has been known for decades [5–8], the complex picture of alternative routes with different cargo proteins using different molecular mechanisms and machineries to get transported into the extracellular space emerged only recently [9–16].

These processes have been collectively termed '**UPS**' (see Glossary), along with a classification into four subtypes that specify different pathways (Figure 1) [14–16]. Unconventional secretion mediated by direct protein translocation across the plasma membrane has been classified as type I UPS [9,14–16]. Another mechanism of UPS associated with the plasma membrane is the translocation of lipidated cargoes driven by ABC transporters, a process that has been termed type II UPS [9,14–16]. While type I and II mechanisms of UPS do not involve membrane-bound compartments as intracellular carriers, type III UPS is based upon vesicular intermediates to transport cargoes into the extracellular space [9,10,14–16]. A number of origins of such vesicles have been implicated in these processes such as autophagosomes, as well as endocytic compartments including secretory lysosomes and multivesicular bodies carrying vesicles that are released into the extracellular space as exosomes. Finally, some types of **integral membrane proteins** are transported from the ER to the plasma membrane in a Golgi-independent manner, a process that is also known as '**Golgi bypass**' and has been designated as type IV UPS [9,16,17]. Another way for proteins to exit cells is mediated by nanotubes; however, in this case, proteins are not secreted into the extracellular space but are transmitted into

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neighboring cells through membrane-bound channels that physically connect cells (Figure 1) [18,19]. The complexity of these pathways has been illustrated in a recent special issue on UPS [13,17,20–26] and several comprehensive review articles [9,15,16,27].

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Beyond the diverse mechanisms being involved in UPS, the range of **cargo proteins** with diverse biological functions is steadily increasing. To expand our knowledge about proteins secreted by unconventional means, bioinformatics tools have been developed to reveal them based on their primary structures [28]. In some cases, bioinformatics tools are available to predict secretion of a given cargo protein through one of the subtypes of UPS, such as unconventional secretion by exosomes [29]. In conclusion, the combination of the molecular analysis of routes of UPS along with comprehensive analyses of the secretomes of a broad range of cell types and organisms revealed the repertoire of secretory mechanisms in mammalian cells to be much more complex than previously assumed.

With many comprehensive review articles available discussing the full range of unconventional secretory processes in lower eukaryotes such as yeast and mammalian cells, the purpose of this article will be to focus on the most well-defined example and to discuss in detail the biochemical basis of this process, the unconventional secretory mechanism of FGF2.

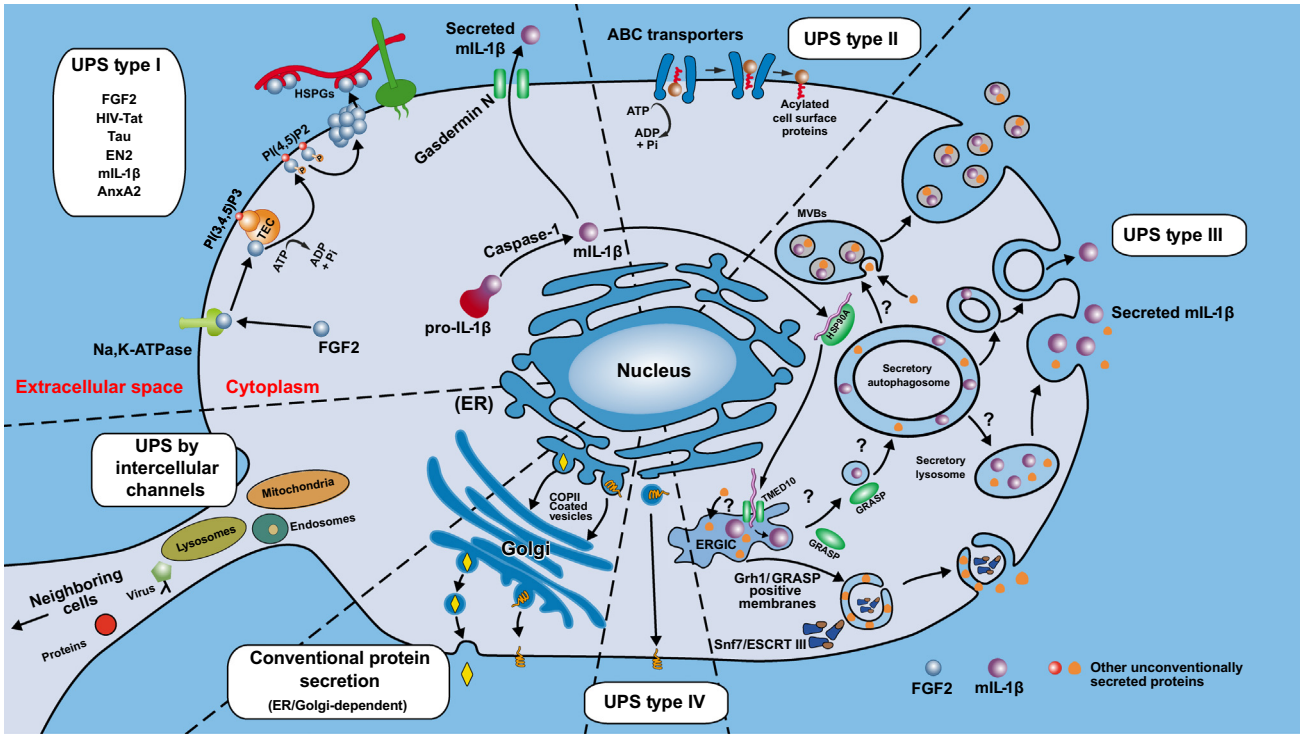

Figure 1. An overview of various mechanisms mediating protein secretion from mammalian cells. Unconventional secretory pathways have been classified into different types that are either based on direct protein translocation across the plasma membrane (UPS type I and type II) or involve intracellular vesicle intermediates such as autophagosomes and endocytic compartments (UPS type III). Variations of the conventional ER/Golgi-dependent secretory pathway that are characterized by a Golgi bypass during transport of integral membrane proteins from the ER to the plasma membrane have been classified as UPS type IV. Proteins and even subcellular organelles can also exit cells using intercellular channels that connect cells and are also known as nanotubes. Abbreviations: COP, coat protein complex; ERGIC, endoplasmic-reticulum–Golgi intermediate compartment; ESCRT, endosomal sorting complexes required for transport; ER, endoplasmic reticulum; FGF, fibroblast growth factor; HSPG, heparan sulfate proteoglycans; Il, interleukin; MVB, multivesicular body; UPS, unconventional protein secretion.

Unconventional secretion mediated by direct protein translocation across plasma membranes

Vesicular mechanisms of UPS (type III and type IV UPS; Figure 1) share a number of features with the conventional ER/Golgi-dependent transport route, such as a requirement for the fusion of membrane-bound compartments [9,10,16,17]. Likewise, ABC transporters are not only involved in unconventional secretory processes (type II UPS; Figure 1) but are also well known in other processes translocating various kinds of peptides and proteins across different kinds of cellular membranes in a broad range of organisms [30–32]. Therefore, the most uncommon variant of unconventional secretory processes in mammalian cells is represented by cargo proteins that are capable of forming lipidic membrane pores for direct self-translocation across the plasma membrane (type I UPS; Figure 1). This is a particularly unusual solution as such a process may cause challenges to cells with a need to maintain the integrity of their outer boundaries toward the extracellular space. As illustrated in Figure 1, proteins unconventionally secreted through a type I UPS mechanism include extracellular factors with crucial functions in fundamental processes of cellular physiology. Some of them have been described to make use of multiple UPS pathways such as interleukin (IL)-1β and Tau, UPS cargoes that can be secreted by both type I and type III mechanisms [15,33].

A common hallmark of the type I UPS pathway is the ability of cargo proteins to physically interact with phosphoinositides or other acidic membrane lipids located in the inner leaflet of the plasma membrane. In particular, unconventional secretion of FGF2 [34–36], HIV-Tat [37], Tau [38], and EN2 [39] depends on the recruitment of these proteins at the inner plasma membrane leaflet mediated by $PI(4,5)P_2$, a phosphoinositide that is strongly enriched in this subcellular location. Other examples, such as AnxA2, are also known to bind to acidic membrane lipids with a role of transbilayer lipid movements as part of **lipidic pores** being proposed recently to drive AnxA2 membrane translocation [40].

Acidic membrane lipids also play a role in Gasdermin-dependent unconventional secretion of IL-1β, a protein that can be secreted unconventionally by both type I and type III UPS. In the case of IL-1β secretion mediated by type I UPS, the role of $PI(4,5)P_2$ is indirect since IL-1β does not physically interact with membrane lipids [41]. However, following the assembly of active inflammasomes in hyperactivated immune cells, the pore-forming activity of an N-terminal fragment of Gasdermin D depends on interactions with acidic membrane lipids including phosphoinositides [42,43]. Therefore, in this case, the pore-forming activity (Gasdermin N) is separated from the cargo protein (IL-1β) to be translocated into the extracellular space. Nevertheless, these observations are in line with a role for $PI(4,5)P_2$ in targeting IL-1β to plasma membrane subdomains mediating unconventional secretion through a type I UPS pathway [44]. In the presence of strong danger signals leading to full activation of the inflammatory response, Gasdermin-dependent membrane pore formation can also lead to pyroptosis, a process that ultimately results in cell death and permits the cellular release of additional cytokines linked to inflammation such as HMGB1 [45]. By contrast, Gasdermin-dependent type I UPS of IL-1β occurs from intact cells along with the possibility of removing the corresponding plasma membrane pores through 'endosomal sorting complex required for transport' (ESCRT)-dependent repair mechanism [46]. However, it is currently unclear how activated inflammasomes can be switched off, so it remains a possibility that immune cells secreting IL-1β in an intact state die at later time points.

A second molecular requirement that is shared at least among some type I UPS cargo proteins is their ability to bind to acidic **heparan sulfate chains** of proteoglycans that are located on cell surfaces. The recognition of the functional relevance of the interaction of FGF2 with heparan

Glossary

'Extracellular trap' hypothesis: a concept proposed to explain the role of cell surface heparan sulfates capturing and disassembling plasma membrane-associated FGF2 complexes as the final step of FGF2 secretion ensuring unidirectional transport of FGF2 across the plasma membrane.

FGF signaling complexes: ternary complexes on cell surfaces consisting of high-affinity FGF receptors, heparan sulfate chains, and FGF2 transmitting mitogenic signals into cells.

Giant unilamellar vesicles (GUVs): artificial membrane vesicles with large diameters (≥10 μm) and a single limiting membrane that can be imaged by confocal microscopy.

Glypican-1 (GPC1): a heparan sulfate proteoglycan that is associated with the plasma membrane via a GPI anchor and contains three O-linked heparan sulfate chains in close proximity to the cell surface.

Golgi bypass: transport phenomena observed for various kinds of integral membrane proteins that are inserted into the ER and get transported to the plasma membrane in a Golgi-independent manner.

Heparan sulfate chains: extended, unbranched chains of O-linked repetitions of disaccharide units made from xylose, galactose, glucuronic acid, N-acetylgalactosamine, iduronic acid as well as sulfated variants of N-acetylgalactosamine and iduronic acid that are contained as post-translational modifications in various kinds of proteoglycans such as glypicans and syndecans.

Integral membrane proteins: membrane-associated proteins containing at least one membrane-spanning hydrophobic element.

Lipidic pores: membrane pores that are not stabilized by integral membrane proteins but rather are induced by the oligomerization of soluble membrane lipid-binding proteins that trigger the formation of pores with a toroidal architecture.

Liquid-ordered nanodomains: small membrane domains that are enriched in cholesterol and sphingomyelin and are characterized by a physical state in which the lateral mobility of membrane components is limited.

Unconventional protein secretion (UPS): pathways of protein secretion from mammalian cells with the secreted

proteins not traveling through the lumen of the ER and the Golgi apparatus.

sulfates [47] has been the original key finding that eventually led to the unraveling of the molecular mechanism by which FGF2 is secreted from cells. Intriguingly, a direct role for heparan sulfates could also be established for unconventional secretion of Tau, a process that mediates transcellular spreading of Tau in the context of neurodegenerative diseases [38,48]. As discussed earlier, a common denominator of type I UPS cargoes is their ability to bind acidic membrane lipids, which makes them in turn candidates to bind heparan sulfates as well. It will therefore be an interesting question for future studies to test whether, like $PI(4,5)P_2$, heparan sulfate chains have a general role in type I UPS pathways of a broad range of cargo proteins.

At least in some cases, further similarities among type I UPS cargoes have been recognized. For example, the Na,K-ATPase is known to play a critical role in unconventional secretion of FGF2 [49–53]. A similar observation has recently been made for HIV-Tat [54]. It has been speculated that the Na,K-ATPase does not only play a role as a landing platform for FGF2 and HIV-Tat at the inner plasma membrane leaflet but may also be modulated in its activity through physical interactions of, for example, FGF2 with its α1 subunit. In fact, FGF2 binds to a region in α1 that contains the ATPase domain of the Na,K-ATPase [49]. This could be important for the maintenance of the plasma membrane potential since, as mentioned earlier, type I UPS cargoes such as FGF2, HIV-Tat, and Tau have been demonstrated to form transiently occurring lipidic membrane pores through which type I UPS occurs [34,35,38,55,56].

The biogenesis of these pores depends on oligomerization which, in the case of FGF2, involves the formation of intermolecular disulfide bridges [55]. Interestingly, cysteine residues required for unconventional secretion have recently also been discovered in Tau [57,58]. A need for oligomerization along with a potential requirement for the formation of disulfides may also be relevant for AnxA2, a type I UPS cargo that appears to translocate across the plasma membrane mediated by a mechanism that involves the formation of a lipidic membrane pore and that is driven by lipid flip flop [40]. It is currently unknown as to whether lipid flip flop is a general phenomenon of UPS type I mechanisms and it is also unclear whether such a process could be compatible with **liquid-ordered nanodomains** enriched in cholesterol, the latter being implicated in unconventional secretion of FGF2 (see later).

FGF2, the most well-defined UPS cargo protein, undergoes self-translocation across the plasma membrane

It has now been known for more than three decades that FGF2 executes extracellular functions linked to its ability to form ternary signaling complexes with heparan sulfates and FGF high-affinity receptors on cell surfaces [59–61]. However, when its primary structure was determined, it became clear that FGF2 does not have a signal peptide for ER/Golgi-dependent protein secretion [8]. While it was then hypothesized that alternative pathways of protein secretion may exist, it took many years until detailed insights into the molecular mechanism by which FGF2 is transported into the extracellular space could be obtained.

The elucidation of the molecular machinery mediating unconventional secretion of FGF2 began with a decisive discovery in 2006 when we found that heparan sulfate chains exposed on cell surfaces do not only engage in **FGF signaling complexes** but also play a direct role in FGF2 secretion [47]. Since FGF2 secretion from heparan sulfate-deficient cells could be rescued by a second population of cells expressing cell surface heparan sulfates that were brought into close proximity forming cell–cell contacts, the '**extracellular trap' hypothesis** was put forward [27]. As illustrated in Figure 2, these findings had the implication that FGF2 is transported into the extracellular space by direct translocation across the plasma membrane.

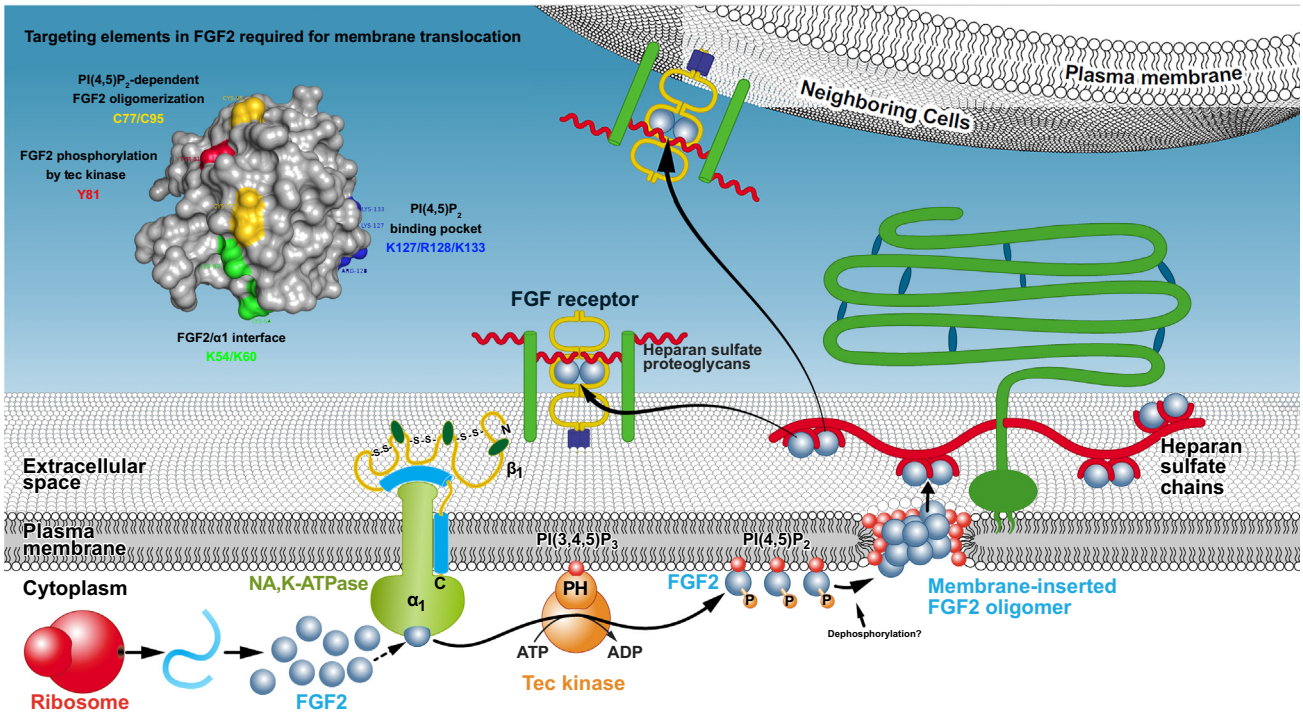

Figure 2. The unconventional secretory pathway of fibroblast growth factor (FGF)2. FGF2 follows a UPS type I pathway that does not involve intracellular vesicle intermediates but rather is mediated by direct translocation of FGF2 across the plasma membrane. This process is initiated at the inner plasma membrane leaflet and involves sequential interactions of FGF2 with the α-subunit of the Na,K-ATPase, Tec kinase, and a membrane lipid, the phosphoinositide PI(4,5)P_2. While the Na,K-ATPase and Tec kinase are auxiliary factors whose precise function is currently unclear, the core mechanism of FGF2 membrane translocation is triggered by PI(4,5)P_2-dependent oligomerization of FGF2. This process leads to the transient formation of lipidic membrane pores accommodating FGF2 oligomers within their hydrophilic centers. Cell surface proteoglycans containing heparan sulfate chains capture and disassemble these translocation intermediates resulting in the exposure of FGF2 on cell surfaces. The *cis* elements in FGF2 such as residues building the binding pocket for PI(4,5)P_2 or residues involved in PI(4,5)P_2-dependent FGF2 oligomerization have been identified. The FGF2 species that are found on the cell surface are most likely dimers that can engage in ternary complexes with heparan sulfates and FGF receptors mediating either autocrine or paracrine signaling.

Another discovery we made shortly thereafter was the ability of FGF2 to physically interact with the phosphoinositide PI(4,5)P_2 [36,62], an acidic membrane lipid enriched in the inner leaflet of the plasma membrane. This interaction was found to trigger FGF2 oligomerization concomitant with the formation of a lipidic membrane pore with a toroidal architecture [35,55]. With these findings combined, physical interaction partners of FGF2 had been identified on both sides of the plasma membrane connected through a lipidic membrane pore. Of note, binding of FGF2 to PI(4,5)P_2 versus heparan sulfates was found to be mutually exclusive with overlapping binding epitopes [34]. The affinity of FGF2 of about 100 nM toward heparin used as a surrogate for heparan sulfate chains [63] is much stronger than that toward PI(4,5)P_2 (~5 μM; [36]). This is why heparan sulfates chains at the outer leaflet are able to outcompete PI(4,5)P_2 with regard to membrane-inserted FGF2 oligomers serving as intermediates in FGF2 membrane translocation. In turn, cell surface heparan sulfates capture and disassemble membrane-inserted FGF2 oligomers, resulting in directional transport of FGF2 across the plasma membrane. Indeed, we could identify the minimal machinery sufficient for FGF2 membrane translocation in a fully reconstituted system based upon **giant unilamellar vesicles (GUVs)** with an inside-out membrane topology [34]. These studies demonstrated that PI(4,5)P_2 on one side and heparan sulfates on the opposing side, along with the ability of FGF2 to oligomerize, constitute the minimal requirements for FGF2 to form a membrane pore through which it can self-translocate across a

membrane (Figure 2). Consistently, FGF2 variants carrying amino acid substitutions that either prevent binding to PI(4,5)P_2, binding to heparan sulfates or FGF2 oligomerization fail to get secreted from cells as well as do not translocate across the membranes of PI(4,5)P_2- and heparin-containing GUVs in biochemical reconstitution experiments [34–36,55]. Recently, using single-molecule high-resolution total internal reflection fluorescence (TIRF) microscopy, we succeeded in the real-time visualization of single events of FGF2 translocation across the plasma membrane in living cells [64]. We observed that this process is amazingly fast with an average time interval of just 200 ms between FGF2 recruitment at the inner leaflet and its appearance at the outer leaflet of the plasma membrane.

The basic mechanism illustrated in Figure 2 was a compelling explanation for earlier studies demonstrating that FGF2 membrane translocation in intact cells does not require an unfolded translocation intermediate [65,66]. Instead, since occurring at the level of the plasma membrane, we interpreted the requirement for FGF2 being folded properly to form defined oligomers that can trigger a membrane pore to represent an intrinsic quality control mechanism ensuring FGF2 secretion to be limited to biologically active molecules [6,7,65,66]. This conclusion was very different from what researchers had anticipated in the early 1990s thinking of a more conventional scenario suggesting that unconventional secretion of FGF2 might be mediated by some sort of a membrane-associated protein-conducting channel using an unfolded FGF2 translocation intermediate as a substrate.

Beyond the core machinery of FGF2 membrane translocation consisting of PI(4,5)P_2 and heparan sulfates on opposing sides of the plasma membrane along with the ability of FGF2 to form pores through oligomerization, additional gene products have been found in a genome-wide RNA interference (RNAi) screen to play a role in unconventional secretion of FGF2 from cells, Tec kinase, and the Na,K-ATPase [52]. Both proteins physically interact with FGF2 and are required for the efficient secretion of FGF2 [52,53,67]. While the precise role of Tec kinase in this process is unknown, the α1 subunit of the Na,K-ATPase has recently been shown to represent the first physical contact of FGF2 at the inner plasma membrane leaflet [49] (Figure 2). This means the interaction with the Na,K-ATPase precedes the interaction of FGF2 with PI(4,5)P_2. This observation led to the hypothesis that the Na,K-ATPase may not only function to direct FGF2 to the inner plasma membrane leaflet but may also exert a quality control mechanism. This is because it was found that the interaction of FGF2 with the Na,K-ATPase is a requirement for the subsequent recruitment of FGF2 by PI(4,5)P_2 [49]. This setting could be the basis for a mechanism by which FGF2 upregulates the activity of the Na,K-ATPase before it is handed over to PI(4,5)P_2, initiating membrane pore formation. In this way, combined with the highly transient nature of the translocation intermediate in intact cells being present for just 200 ms [64], a potential harm to the membrane potential at sites of FGF2 membrane translocation could be prevented. A possible mechanism could be that the interaction with the Na,K-ATPase causes FGF2 to dimerize, an increase in avidity that may be required for efficient recruitment of FGF2 by PI(4,5)P_2.

GPC1 is a GPI-anchored heparan sulfate proteoglycan and a key driver of FGF2 secretion

Cell surface heparan sulfate chains are linked to membrane-associated core proteins to form heparan sulfate proteoglycans. These belong to different families, with syndecans and glypicans being the two most abundant classes. With regard to the role of heparan sulfates in FGF2 secretion [47], it was long thought that their essential role in this process does not depend on the type of proteoglycan they are attached to. Contradictory to this hypothesis, we recently observed that the principal heparan sulfate proteoglycan involved in FGF2 secretion is **GPC1** [68] a member of the glypican family [69]. GPC1 was identified through a BioID screen demonstrating its proximity

to FGF2 in intact cells [68]. Despite the continued presence of normal levels of total heparan sulfates and glycosaminoglycan chains, GPC1 knock-out cell lines did not secrete FGF2 efficiently, though overexpression of GPC1 rescued GPC1 knock-out cells and caused a substantial increase in FGF2 secretion efficiency above wild-type cells [68]. These findings support that GPC1 is a rate-limiting component of the FGF2 secretion machinery. It could further be shown that, as opposed to other heparan sulfate proteoglycans, the heparan sulfate chains of GPC1 are enriched with trisulfated disaccharide units known to mediate high-affinity binding of FGF2 [70]. By contrast, GPC1 was found to be dispensable for FGF2 signaling [68]. The combined findings of this study suggested that, while other heparan sulfate proteoglycans are capable of supporting FGF2 signaling, GPC1 is required for the efficient secretion of FGF2 being the rate-limiting factor of this process [68].

Since GPC1 is a GPI-anchored heparan sulfate proteoglycan without an intracellular domain, we were surprised to find that the interaction with FGF2 could be caught in a cellular BioID screen [68]. Intriguingly, the heparan sulfate chains of GPC1 are linked to the core protein in an atypical manner compared with other heparan sulfate proteoglycans [69,71]. They are located in a membrane-proximal manner only about 3 nm away from the GPI anchor of GPC1. GPC1 is further characterized by a lid structure made from the core protein that is located above its membrane-proximal heparan sulfate chains (Figure 3). This spatial arrangement is highly suggestive of a microenvironment directly above the membrane that is shielded by the GPC1 lid in which the GPC1 heparan sulfate chains are highly concentrated and in direct proximity to the outer plasma membrane leaflet. This spatial organization, along with the high-affinity binding sites for FGF2 positioned at the outer leaflet, we feel, is likely to be crucial for the role of GPC1 in capturing and resolving FGF2 translocation intermediates that are inserted into the plasma membrane from the cytoplasmic side (Figure 2). The increase in FGF2 secretion efficiencies observed upon overexpression of GPC1 further suggests that its FGF2 binding sites are required already for the efficient membrane insertion of FGF2 oligomers [68]. A scenario appears to be plausible in which productive FGF2 oligomer insertion from the cytoplasmic side depends on the availability of the GPC1 heparan sulfate chains at the outer leaflet to prevent such insertion events from collapsing back into the cytoplasm. Thus, the highly dynamic process of $PI(4,5)P_2$-dependent FGF2 oligomerization and membrane insertion at the cytoplasmic leaflet might be directly coupled to a capturing mechanism at the outer leaflet mediated by GPC1. This in turn would be a compelling explanation why GPC1 overexpression strongly enhances FGF2 secretion efficiencies [68].

The role of GPC1 in FGF2 secretion raises further questions on how the two leaflets of the plasma membrane are coupled during FGF2 membrane translocation. In other words, how does FGF2 find the correct locations at the inner plasma membrane leaflet with GPC1 on the opposing side of the membrane? A reasonable hypothesis would be that the machinery components known to be required for FGF2 membrane translocation do not associate with each other through random diffusion but rather are preorganized in membrane nanodomains. Specifically, we proposed the existence of membrane nanodomains that contain a subpopulation of the Na,K-ATPase and $PI(4,5)P_2$ at the inner plasma membrane leaflet as well as GPC1 in the outer plasma membrane leaflet (Figure 3) [68]. Since the GPI anchor of GPC1 partitions into domains enriched in cholesterol, we further hypothesized that all components required for FGF2 membrane translocation are organized together in liquid-ordered plasma membrane nanodomains. Indeed, in a recent study, a role for cholesterol promoting the efficient recruitment of FGF2 to the inner leaflet could be established [72]. Beyond biochemical reconstitution experiments and molecular dynamics simulations to study the mechanism by which cholesterol promotes both headgroup visibility and clustering of $PI(4,5)P_2$, this study also demonstrated that increased levels of cholesterol in the plasma membrane promote increased FGF2 secretion efficiencies. Intriguingly, unconventional

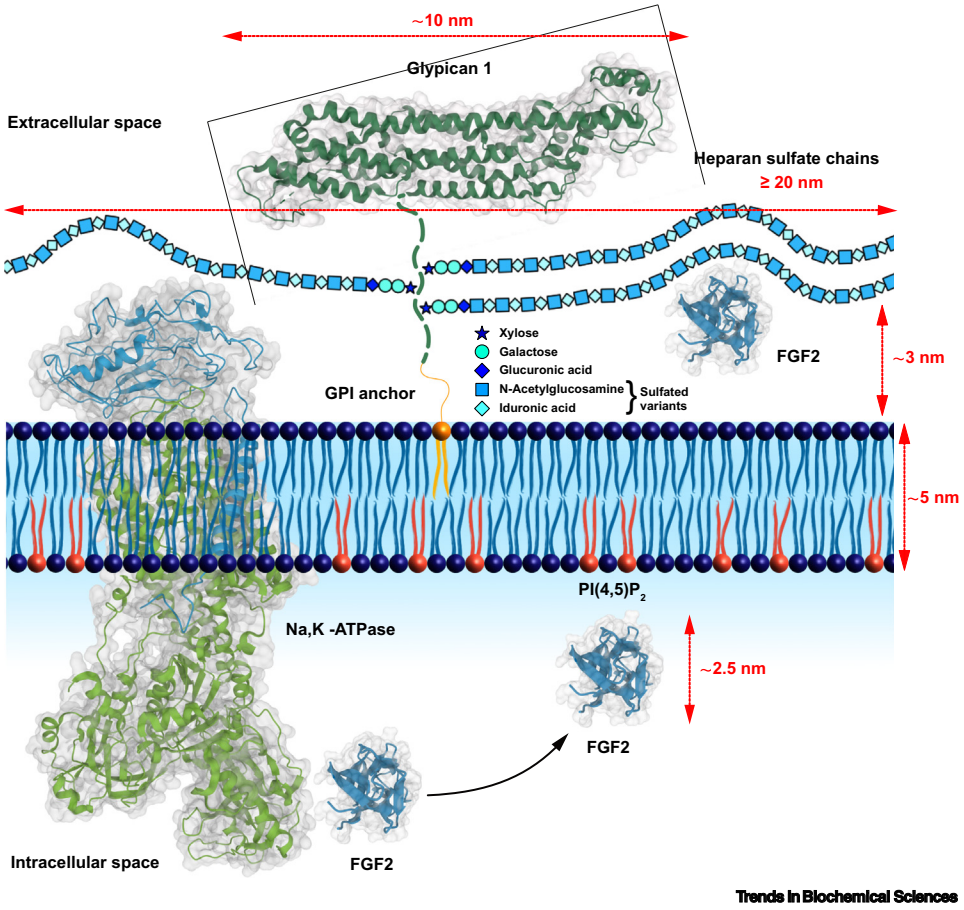

Figure 3. Glypican-1 (GPC1), a heparan sulfate proteoglycan that drives unconventional secretion of FGF2. In a recent study, GPC1 was identified as a heparan sulfate proteoglycan having a critical function for the efficient secretion of fibroblast growth factor (FGF)2 [68]. Its heparan sulfate chains contain high-affinity binding sites for FGF2. Furthermore, they are arranged in a way bringing them in close proximity to the extracellular surface of the plasma membrane. As illustrated in this figure, the heparan sulfate chains of GPC1 are arranged in a way with a distance of only about 3 nm away from the plasma membrane. We propose that GPC1 with its proteinaceous lid structure and its heparan sulfate chains beneath the lid forms a microenvironment right above the plasma membrane that is specialized in capturing and disassembling FGF2 oligomers on the cell surface. PDB IDs: GPC-1 - 4YWT; FGF2 – 1BFF; Na,K-ATPase – 3KDP.

secretion of Tau, another type I UPS cargo protein (Figure 1), has been demonstrated to depend on cholesterol-enriched plasma membrane domains, as well [48]. Based on these considerations, it will be an interesting aspect of future studies to challenge the hypothesis of membrane nanodomains coordinating FGF2 membrane translocation using high-resolution imaging techniques.

Biomedical implications of the intimate relationship of GPC1 and FGF2 in type I UPS

Unconventional secretory processes mediate transport into the extracellular space of cargo proteins with fundamental functions in cellular physiology in health and disease [12,15,16,33]. Among them are factors involved in cancer cell survival and tumor-induced angiogenesis such as FGF2, viral replication such as HIV-Tat, neurodegeneration such as Tau, and chronic inflammation such as IL-1β, among many other examples. In some cases, proteome-wide analyses have even revealed a general role of UPS as a major contributor to the spectrum of secretory proteins when benign cells turn into cancer cells [73].

FGF2 has major clinical significance in malignancies of a broad range of cancer entities including both solid and hematological tumors [74]. Excessive mitogenic signaling induced by FGF2 as part of a ternary complex with heparan sulfates and high-affinity FGF receptors promotes tumor progression. Furthermore, FGF2 signaling increases the angiogenic potential in a way that can lead to metastatic cancer phenotypes. Dysregulated FGF2 signaling is also known to cause chemoresistances in the context of established cancer therapies leading to even more aggressive clones of cancer cells resulting in poor clinical outcomes. A prime example is acute myeloid leukemia (AML) with approved drugs such as FLT3 (FMS-like tyrosine kinase 3) inhibitors that efficiently kill AML-derived cancer cells; however, in a highly specific manner, FGF2 signaling blocks the cytotoxic effects of FLT3 inhibitors producing resistant AML clones [75,76]. The underlying mechanism is based upon a signaling cascade that mediates tumoral immune escape by activating a FGF2-dependent cell survival pathway [77]. The ability of FGF2 to act as a survival factor for AML-derived cancer cells has been shown to be nonredundant, that is, other members of the FGF family or other growth factors are incapable of promoting cell survival of AML cells [75,76]. Therefore, the unconventional secretory mechanism used by FGF2 holds great promise as a druggable pathway to develop novel types of drugs that can be useful in combination with FLT3 inhibitors to treat AML.

The recent discovery of GPC1 being the key driver of unconventional secretion of FGF2 identifies a so far unknown component of the FGF2 membrane translocation machinery that, in addition to other factors such as PI(4,5)P_2 and the Na,K-ATPase, could serve as a druggable target to prevent tumor cells and their microenvironment from secreting FGF2 [68]. Intriguingly, GPC1 itself is known to modulate various signaling pathways induced by FGF family members, vascular endothelial growth factor (VEGF)-A, transforming growth factor (TGF)-β, among others, and is strongly overexpressed in a broad range of malignancies such as pancreatic cancer, glioblastoma, and uterine cervical cancers [69]. On top of these functions in cancer-related signaling pathways, it is now recognized that GPC1 also plays a direct role in the extracellular availability of FGF2 as part of its unconventional pathway of secretion [68]. Thus, a so far unknown intimate relationship between FGF2 and GPC1 has been discovered that has broad relevance for future strategies to block the biological function of FGF2 under pathophysiological conditions.

Concluding remarks

A number of outstanding questions about the molecular mechanism and machinery mediating the type I UPS pathway of FGF2 remain to be addressed in future studies (see Outstanding questions). For example, our understanding of how PI(4,5)P_2-dependent FGF2 oligomerization results in membrane remodeling producing a lipidic membrane pore with a toroidal architecture remains unknown. In this context, it is also a major question as to whether the asymmetric distribution of PI(4,5)P_2 across the two leaflets of the plasma membrane is relaxed with PI(4,5)P_2 appearing in the outer leaflet as part of FGF2 membrane translocation. If so, this phenomenon could represent a thermodynamic driving force facilitating unconventional secretion of FGF2. Since other type I UPS cargo proteins such as HIV-Tat, Tau, and EN2 bind to PI(4,5)P_2 as well [37–39,48], do they also oligomerize to form membrane pores? Similar to FGF2, cysteine residues have recently been shown to be important *cis* elements in unconventional secretion of Tau [57,58]. Are they required to promote oligomerization of Tau which, in turn, leads to membrane pore formation?

The precise role of the Na,K-ATPase in unconventional secretion of FGF2 remains a mystery as well. As hypothesized earlier, its role might be related to the maintenance of the membrane potential under conditions of type I UPS events that are mediated by transient membrane pores. Indeed, the Na,K-ATPase is also involved in unconventional secretion of HIV-Tat [54]. Do all

Outstanding questions

What are the structural determinants that permit FGF2 to form a lipidic membrane pore through PI(4,5)P_2-dependent oligomerization?

Does the asymmetric distribution of PI(4,5)P_2 constitute a thermodynamic driving force facilitating FGF2 membrane translocation?

Does FGF2 binding to the Na,K-ATPase modulate its activity to maintain the membrane potential during FGF2 membrane translocation?

Is the molecular machinery mediating FGF2 translocation across the plasma membrane organized in a nanodomain structure with the Na,K-ATPase, PI(4,5)P_2, and GPC1 being located in cholesterol-enriched liquid-ordered membrane domains?

Are cell surface heparan sulfate chains linked to GPC1 required for type I UPS of other cargo proteins such as HIV-Tat, Tau, and EN2?

Do additional factors exist that are required for type I UPS of FGF2 such as redox enzymes that transfer electrons to so far unknown acceptors during the formation of disulfides in PI(4,5)P_2-dependent FGF2 oligomerization? Are such factors also relevant for type I UPS of other cargo proteins such as HIV-Tat, Tau, and EN2?

Does type I UPS of FGF2 represent a druggable pathway that can be exploited to develop inhibitors that block the biological function of FGF2 under pathophysiological conditions such as cancer cell survival and tumor-induced angiogenesis?

type I UPS cargo proteins depend on the activity of the Na,K-ATPase? Similarly, are there in addition to FGF2 other type I UPS cargo proteins such as Tau whose secretion depends on cell surface heparan sulfate chains?

The answers to these and other questions will not only be important to obtain a detailed understanding of how FGF2 is secreted from cells but rather will also help to resolve the mechanism of unconventional secretion of HIV-Tat, Tau, and EN2, among other potential type I UPS cargoes. In addition, as we understand more about the underlying mechanisms of these unusual pathways of protein secretion opportunities will surface to develop novel kinds of useful drugs. With the role of HIV-Tat in viral replication, the role of Tau in neurodegenerative diseases, and the ability of FGF2 to cause chemoresistances that limit the potency of FLT3 inhibitors in the treatment of acute myeloid leukemia, unconventional secretory pathways as druggable targets hold great promise for the development of novel therapies to fight cancer, viral infections, and neurodegenerative disorders, among other diseases.

Declaration of interests

No interests are declared.

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