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Review

The Unconventional Secretory Machinery of Fibroblast Growth Factor 2

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Unconventional secretory proteins represent a subpopulation of extracellular factors that are exported from eukaryotic cells by mechanisms that do not depend on the endoplasmic reticulum and the Golgi complex. Various pathways have been implicated in unconventional secretion including those involving intracellular membrane-bound intermediates and others that are based on direct protein translocation across plasma membranes. Interleukin 1β (IL1β) and fibroblast growth factor 2 (FGF2) are classical examples of unconventional secretory proteins with IL1β believed to be present in intracellular vesicles prior to secretion. By contrast, FGF2 represents an example of a non-vesicular mechanism of unconventional secretion. Here, the author discusses the current knowledge about the molecular machinery being involved in FGF2 secretion. To reveal both differential and common requirements, this review further aims at a comprehensive comparison of this mechanism with other unconventional secretory processes. In particular, a potentially general role of tyrosine phosphorylation as a regulatory signal in unconventional protein secretion will be discussed.

Key words: AcbA, Acb1, acyl-CoA-binding protein, annexin A2, fibroblast growth factor 2, heparan sulfate proteoglycans, HIV Tat, interleukin 1β, non-classical export, phosphoinositides, PI(4,5)P_2, unconventional protein secretion

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Around 20 years ago in the early 1990s, following the identification of a number of seemingly unrelated examples of soluble extracellular factors without hydrophobic signal sequences, unconventional protein secretion was first recognized as a potentially general phenomenon (1). Among the first cases were extracellular factors such as interleukin 1β (IL1β) (2), galectin 1 (3) as well as fibroblast growth factor (FGF) 1 (4) and FGF2 (5,6), proteins that are now well established as classic examples of unconventional secretory proteins (7,8). Ever since, the list of proteins suggested to exit cells in an endoplasmic reticulum (ER)/Golgi-independent manner has been steadily growing and now, among others, includes proteins such as annexins (9), thioredoxin (10), high mobility group box (HMGB) proteins (11–14), acyl-CoA-binding protein (15–17) as well as viral proteins with HIV Tat being the most prominent examples (18,19).

Unconventional secretion occurs in both constitutive and regulated modes. For example, galectin-1 and FGF2 are secreted from a wide range of cells without any requirement of an external stimulus. By contrast, secretion of IL1β from monocytes depends on triggers such as bacterial lipopolysaccharides as part of the inflammatory response. Other typical triggers of unconventional secretory processes are various kinds of stresses, a typical example being exposure of cells to elevated temperature that leads to the secretion of FGF1 (4). Stress conditions also provide a link to unconventional secretory processes in lower eukaryotes as prolonged starvation has been shown to cause secretion of acyl-CoA-binding protein from yeast (Acb1) and *Dictyostelium* (AcbA) (15–17).

Some unconventional secretory proteins appear to function primarily, if not exclusively, in the extracellular space with high-affinity cell surface receptors known for IL1β (20,21) as well as FGF1 and FGF2 (22–24). By contrast, besides their functions on cell surfaces where they are bound to β-galactosides (25,26), galectins have also been implicated in intracellular processes (27,28). Finally, many unconventional secretory proteins were long thought to function exclusively inside cells; however, proteins such as thioredoxin (10,29), HMGB proteins (11,12) or AcbA/Acb1 (15–17,30) were later found to get exported from cells under certain physiological conditions. In the extracellular space, they appear to serve distinct functions with the extracellular role of the chromatin-binding protein HMGB1 as a cytokine being a prominent example (11–14,31).

With regard to mechanisms involved in ER/Golgi-independent protein secretion, two general types can be distinguished. One group of unconventional secretory proteins is characterized by the ability to interact with membrane lipids at the inner leaflet of plasma membranes (32–34). As discussed below, these interactions are believed to result in the formation of homo- or hetero-oligomeric complexes that may insert transiently into membranes followed by their release into the extracellular space. This type of unconventional secretion has been defined as a non-vesicular mechanism and proteins

such as FGF1 and FGF2, annexin A2 as well as HIV Tat are probably secreted in this way. A second type of unconventional protein secretion collectively refers to mechanisms that involve intracellular transport vesicles that are not derived from the ER/Golgi system (7,8). Three types of such membrane-bound intermediates have been discussed as playing a role in unconventional secretion. These are secretory lysosomes, microvesicles forming at cell surfaces and exosomes present in multivesicular bodies. All three have been implicated in regulated secretion of IL1β (33). Another example is Acb1/AcbA, whose secretion was proposed to involve multivesicular bodies (15,17,30,33). In this case, initial capturing is thought to be mediated by autophagosomes that, in turn, fuse with multivesicular bodies (15,17,30). For other unconventional secretory proteins such as galectins, thioredoxin and HMGB1, it is less clear whether they are secreted by non-vesicular or vesicle-dependent mechanisms of unconventional secretion.

The Unconventional Secretory Machinery of FGF2

For many years following its discovery in the late 1980s (5,6,35–37), the mechanistic aspects of the unconventional secretory route of FGF2 remained elusive (38). Recently, however, insight into molecular components and mechanistic details of the secretory machinery of FGF2 has been gained (7,8,33). A first key observation was made with an *in vitro* system using affinity-purified plasma membrane inside–out vesicles that expose the cytoplasmic leaflet on their surfaces. In these experiments, FGF2 was found to be capable of traversing plasma membranes in a directional manner accumulating in the lumen of such vesicles (39,40). These findings suggested that the unconventional secretory route of FGF2 does not involve intracellular membrane-bound intermediates but rather represents some type of direct protein translocation across the plasma membrane. On the basis of a *C*-terminal cluster of basic amino acids, FGF2 was then identified as a phosphoinositide-binding protein (8,41). Using a novel flow cytometry assay to study protein–lipid interactions (42), FGF2 was shown to bind to $PI(4,5)P_2$ with high specificity. This interaction mediates recruitment of FGF2 at the inner leaflet of plasma membranes and was shown to be essential for FGF2 membrane translocation. Reduction of cellular $PI(4,5)P_2$ levels induced by RNAi-mediated downregulation of PIP kinases caused a substantial drop in FGF2 secretion efficiency. Likewise, FGF2 variant forms that cannot bind $PI(4,5)P_2$ were impaired with regard to secretion rates. These findings establish that $PI(4,5)P_2$-mediated recruitment of FGF2 represents an essential step in the overall process of FGF2 secretion (8,41).

On the basis of the *in vitro* reconstitution experiments discussed above it has been concluded that, following binding to the cytoplasmic leaflet, FGF2 translocates to the extracellular side of plasma membranes (39,40). Transporters such as classical protein-conducting channels or ABC transporters have been considered to play a role in this process; however, no experimental evidence is available for this hypothesis. Alternatively, it has been speculated that $PI(4,5)P_2$-dependent recruitment at the inner leaflet of plasma membranes may cause FGF2 to oligomerize followed by membrane insertion (8,33). This idea is supported by the findings showing that binding of heparin or heparan sulfates enhances self-oligomerization of FGF2 (43–47). As the binding site for heparin overlaps with the binding site for $PI(4,5)P_2$ as part of the *C*-terminal basic cluster in FGF2 (41), it seems reasonable to assume that FGF2 can form $PI(4,5)P_2$-induced dimers and possibly higher oligomers already inside cells. Therefore, as an alternative to the transporter hypothesis, it has been speculated that $PI(4,5)P_2$-induced oligomerization may cause FGF2 to penetrate plasma membranes as a key mechanism of membrane translocation.

To further discriminate between transporter-dependent and membrane insertion-mediated translocation models of unconventional secretion, the folding state of FGF2 during membrane translocation was investigated. The rationale for this was that all known examples of protein-conducting channels analyzed at the molecular level such as Sec61 of the ER (48) or the TIM/TOM machineries of the inner and outer membranes of mitochondria (49) as well as protein-conducting ABC transporters (50) require cargo proteins to be largely unfolded during membrane translocation. By contrast, a mechanism of membrane translocation that is based on oligomerization and membrane insertion is probably based on the defined protein conformations. Indeed, FGF2 membrane translocation occurs in a folded conformation (33,51,52), a finding that argues against a potential role for a classical protein-conducting channel or ABC transporter in FGF2 membrane translocation. Rather, in this sense, FGF2 secretion may be related in some way to other membrane translocation processes in which proteins traverse membranes in a folded conformation. This is true for protein import into peroxisomes (53,54) and the twin arginine protein translocation systems in bacteria and plants (55,56), processes for which the exact mode of protein translocation across the membrane is not understood at the molecular level.

Although the molecular mechanism of how FGF2 physically traverses the plasma membrane remains unclear, heparan sulfate proteoglycans have been identified as essential factors at the extracellular side of plasma membranes being required to transfer FGF2 into the extracellular space (57,58). This was shown by analyzing FGF2 secretion from cells that cannot synthesize heparan sulfate proteoglycans and by measuring secretion rates of FGF2 mutant forms that cannot bind to heparan sulfates. In both cases, when the interaction between FGF2 and heparan sulfates was prevented, a block in secretion

was observed (58). Interestingly, FGF2 secretion could be rescued when heparan sulfate-deficient cells expressing FGF2 (donor cells) were cocultivated with heparan sulfate-expressing cells that cannot make FGF2 (acceptor cells). Under these experimental conditions, intercellular transfer of FGF2 was observed, resulting in the accumulation of FGF2 on cell surfaces of the acceptor cells where it was bound to heparan sulfates. Intriguingly, this rescue was dependent on the distance between the two cell populations showing that heparan sulfate proteoglycans are required in a membrane proximal orientation to drive FGF2 membrane translocation. This observation confirms direct translocation across plasma membranes as the mechanism of FGF2 secretion. These findings defined heparan sulfate proteoglycans as FGF2 export receptors and led to the molecular trapping hypothesis proposing multiple roles for heparan sulfate proteoglycans in FGF2 secretion, storage on cell surfaces and the extracellular matrix as well as in FGF2 signaling (57). In conclusion, the overall process of unconventional secretion of FGF2 relies on direct translocation of folded FGF2 molecules across the plasma membrane with differential requirements for $PI(4,5)P_2$ at the inner leaflet and heparan sulfate proteoglycans at the extracellular side to drive directional transport of FGF2 into the extracellular space.

How to Identify Additional Molecular Components of the FGF2 Secretion Machinery?

As discussed above, substantial insight into various aspects of the unconventional secretory route of FGF2 has been gained; however, the molecular mechanism by which FGF2 physically traverses the plasma membrane remains a mystery. Likewise, it is unclear whether the biological functions of FGF2 are regulated at the level of secretion. This is because FGF2 secretion has so far been reported as a constitutive process (38,59), with the physiological functions of FGF2 being controlled at the expression level and/or at the level of its mobilization from storage sites on cell surfaces and the extracellular matrix. However, recent evidence suggests that FGF2 secretion from primary skin-derived fibroblasts can be stimulated by activation of the inflammasome (60). It remains to be established whether this latter case represents a cell type-specific phenomenon or whether the biological activity of FGF2 can generally be regulated at the level of secretion. In any case, to address these questions, it is clear that additional insight into molecular components involved in the overall process of FGF2 secretion is required. To conduct a comprehensive and unbiased analysis to identify gene products involved in FGF2 secretion from human cells, a genome-wide screening approach using siRNA arrays was conducted (61). For this purpose, a classic FGF2 secretion assay that is based on flow cytometry (36) was adapted to a multiwell screening platform allowing for the analysis of all gene products known in the human genome.

The first gene product that was identified with this approach turned out to be Tec, one of the several hundred kinases known in the human genome. Tec kinase is the eponymous member of the Tec family of non-receptor tyrosine kinases (62). They are expressed in a wide range of vertebrate tissues; however, the best characterized members of this protein family, Itk and Btk, are found primarily in cells of the hematopoietic lineage and play critical roles in B- and T-cell development and function (62,63). Tec kinases act downstream of various kinds of receptors and are activated by tyrosine phosphorylation which is catalyzed by Src kinases. Following activation, they are recruited to the inner leaflet of plasma membranes mediated by their pleckstrin homology (PH) domains that bind to phosphoinositides (62).

The identification of a PH domain containing a kinase with a putative role in FGF2 secretion was an intriguing finding as FGF2 itself binds to phosphoinositides at the inner leaflet of plasma membranes, a key step in the overall process of FGF2 secretion (see above). In addition to siRNA-mediated downregulation, a pharmacological inhibitor of Tec kinase, LFM-A13, also impaired FGF2 secretion. This result implied that indeed the enzymatic activity of Tec kinase is required in some way to support FGF2 secretion. On the basis of biochemical experiments using purified components, a direct interaction between Tec kinase and FGF2 could be established that results in phosphorylation of FGF2 at tyrosine 82. This modification plays a direct role as substitution with alanine caused a block of FGF2 secretion. By contrast, substitution of tyrosine 82 with a phosphomimetic residue did support FGF2 secretion in a Tec kinase-independent manner. These results were confirmed in a physiologically relevant cell-based assay establishing a role for Tec kinase in FGF2 secretion-dependent proliferation of mouse fibroblasts (61).

It is currently unclear in which way Tec kinase-mediated tyrosine phosphorylation is required for FGF2 transport into the extracellular space. Substitution of tyrosine 82 by alanine causes a block in FGF2 secretion. The other way around, a phosphomimetic residue in this position rescues FGF2 export from cells as well as renders FGF2 secretion independent of Tec kinase (61). These findings establish that phosphorylation of FGF2 at tyrosine 82 is essential for the overall process of FGF2 secretion. On the basis of the knowledge available for the typical activation modes of Tec kinases, a conservative guess would be that FGF2 becomes phosphorylated at the inner leaflet of plasma membranes. This is because, upon activation through tyrosine phosphorylation, Tec kinases redistribute to the cytoplasmic leaflet where their PH domains are recruited by phosphoinositides (62,63). Thus, in turn, tyrosine phosphorylation of FGF2 might only occur when both proteins are brought into proximity in specialized domains containing phosphoinositides with a direct impact of this modification on the mechanism of FGF2 membrane translocation. This may result in

the secretion of FGF2 species that are phosphorylated at tyrosine 82.

An alternative model is based on the assumption that FGF2 is only transiently phosphorylated as part of an essential step preceding FGF2 membrane translocation. For example, Tec kinase may phosphorylate FGF2 already in the cytoplasm. Even though tyrosine-phosphorylated and, therefore, active Tec kinases are thought to primarily localize to plasma membranes, a soluble form of Tec kinase lacking the PH domain can both bind and phosphorylate FGF2 *in vitro* (61). In this way, tyrosine phosphorylation of FGF2 may serve as a signal for FGF2 transport into the cell periphery. Such a mechanism might be related to the phosphorylation of transit peptides as part of the guidance complex-dependent mechanism of protein targeting to plant chloroplasts (64,65).

Differential versus Common Requirements of Unconventional Secretion of Various Cargo Proteins

As discussed above, the phenomenon of unconventional protein secretion does not appear to be based on a single common pathway for all cargo proteins but rather seems to represent a collection of distinct secretory mechanisms (33). Prior to secretion, some cargo molecules are contained in various kinds of intracellular vesicles with IL1β (66–68) and AcbA/Acb1 being well-characterized examples (15,17,69). Recently, a new role for autophagosomes in this type of unconventional secretion has been shown for AcbA/Acb1 secretion (15,17), a pathway that might probably further involve multivesicular bodies and that may also be relevant for IL1β secretion (15,17,30,70). By contrast, FGF2 belongs to a class of unconventional secretory proteins that, prior to secretion, do not appear to be enclosed in intracellular vesicles. Rather, FGF2 binds to the inner leaflet of plasma membranes followed by membrane translocation into the extracellular space. Besides FGF2, other examples of this general type of unconventional secretion are probably FGF1 (34,71,72), HIV Tat (19,73,74) and annexins (75–77), with annexin A2 being the best characterized example. A common denominator of these proteins is their ability to get recruited by membrane lipids as they interact with either phosphoinositides or other acidic phospholipids such as phosphatidylserine. In case of FGF2, $PI(4,5)P_2$-dependent recruitment to plasma membranes has been defined as an essential step in FGF2 membrane translocation (41), a process that has been proposed to involve the formation of FGF2 homo-oligomers (7,33). Similarly, HIV Tat has been shown as a $PI(4,5)P_2$-binding protein, an interaction that is again essential for its secretion (19). Likewise, both FGF1 and annexin A2 have been shown to bind to $PI(4,5)P_2$ and/or phosphatidylserine (78–80). Furthermore, both FGF1 and annexin A2 are contained as dimers in hetero-oligomeric complexes that are recruited at the cytoplasmic leaflet of plasma membranes (71,81,82). Finally, both FGF1 and annexin A2 complexes contain distinct members of the S100 family of calcium-binding proteins that are critical for their translocation to cell surfaces (9,34,72).

Besides these similarities, many questions remain such as a potential role of heparan sulfate proteoglycans in unconventional secretory processes other than FGF2 secretion. This question is particularly relevant for FGF1 and HIV Tat as both proteins are known to bind heparan sulfates (18,34,72). The other way around, it remains unclear whether molecular requirements established for FGF1 and annexin A2, such as the role of S100 proteins, also play a role in FGF2 secretion. Clearly, however, the most critical aspect in the molecular analysis of these unconventional secretory processes remains the mechanism by which FGF1, FGF2, HIV Tat and annexin A2 physically traverse the plasma membrane to reach the extracellular space. As discussed above for FGF2, the formal possibility of protein-conducting channels in the plasma membrane is rather unlikely as they typically carry unfolded proteins only. Alternatively, some way of membrane insertion has been discussed and indeed, experimental evidence is available that annexin A12 is capable of penetrating membranes as shown by spin labeling experiments (83). Although it is unknown as to whether other annexins such as annexin A2 have similar properties, this finding may indicate a potential general role of membrane-inserted intermediates in unconventional protein secretion including cargos such as FGF1, FGF2 and HIV Tat.

Tyrosine Phosphorylation – A General Signal for Unconventional Secretion?

Tec kinase-mediated tyrosine phosphorylation has been shown to be required for unconventional secretion of FGF2 (61). Similar to what has been considered in the previous section one may wonder whether this phenomenon is a peculiar exception of the secretory machinery of FGF2 or whether tyrosine phosphorylation might represent a general signal in unconventional secretion. Indeed, at least three other unconventional secretory proteins have been experimentally shown to undergo tyrosine phosphorylation, annexin A1 (84) and A2 (9,85,86) as well as galectin-3 (87). Although it is unknown whether this modification is relevant for the secretion of annexin A1 and galectin-3, direct evidence has been reported for a role of tyrosine phosphorylation of annexin A2 in membrane translocation of the annexin A2-S100A10 heterotetramer to cell surfaces (9). Thus, striking similarities exist between the secretory mechanisms of FGF2 and annexin A2 in that both proteins bind to $PI(4,5)P_2$ at the inner leaflet concomitant with homo- and/or hetero-oligomerization, are secreted by direct translocation across plasma membranes and make use of tyrosine phosphorylation as a signal for membrane translocation.

In conclusion, following many years without a clear picture, insight into the molecular mechanisms of unconventional secretory processes is emerging. This is true for both vesicular and non-vesicular modes of unconventional secretion and it can be expected that both differential and common requirements along with the molecular mechanisms being involved can be delineated in the years to come.

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