An emerging case for membrane pore formation as a common mechanism for the unconventional secretion of FGF2 and IL-1β

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Abstract

Extracellular proteins with important signalling roles in processes, such as inflammation and angiogenesis, are known to employ unconventional routes of protein secretion. Although mechanisms of unconventional protein secretion are beginning to emerge, the precise molecular details have remained elusive for the majority of cargo proteins secreted by unconventional means. Recent findings suggest that for two examples of unconventionally secreted proteins, interleukin 1β (IL-1β) and fibroblast growth factor 2 (FGF2), the common molecular principle of pore formation may be shared. Under specific experimental conditions IL-1β and FGF2 secretion are triggered by phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2)-dependent formation of pores across the plasma membrane. However, the underlying mechanisms are different, with FGF2 known to directly interact with PI(4,5)P2, whereas in the case of IL-1β secretion, it is proposed that the N-terminal fragment of gasdermin D interacts with PI(4,5)P2 to form the pore. Thus, although implemented in different ways, these findings suggest that pore formation may be shared by the unconventional secretion mechanisms for FGF2 and IL-1β in at least some cases. In this opinion article, we discuss the unconventional mechanisms of FGF2 and IL-1β release with a particular emphasis on recent discoveries suggesting the importance of pore formation on the plasma membrane.
Introduction

Interleukin-1β (IL-1β) and fibroblast growth factor 2 (FGF2) are two of the best studied, and arguably amongst the most important proteins that are unconventionally secreted from mammalian cells. IL-1β is a pro-inflammatory cytokine that is important for host-responses to infection and has also been implicated in the pathogenesis of major human diseases (Dinarello and van der Meer, 2013). IL-1β elicits its effects through activation of the IL-1 receptor on responsive cells, resulting in activation of NF-κB and its downstream pro-inflammatory signalling cascades (Dinarello and van der Meer, 2013). FGF2 is a pro-angiogenic factor that is important in development, but also implicated in tumorigenesis (Akl et al., 2016; Beenken and Mohammadi, 2009). In addition to exerting a pro-angiogenic effect, FGF2 is also a tumour-cell survival factor (Akl et al., 2016; Pardo et al., 2006; Sugimoto et al., 2016), an effect which is mediated by an autocrine FGF2 secretion-signalling loop that prevents tumour cells from undergoing apoptosis. This mechanism is believed to cause resistance to anti-cancer therapies (Akl et al., 2016; Noh et al., 2014). Conventional protein secretion accounts for the majority of secreted proteins and relies upon the presence of an N-terminal signal peptide that directs their trafficking to the ER. Once inserted into the ER, the signal peptide is cleaved and the secretory protein is subsequently trafficked through the ER-Golgi pathway to the outside of the cell through vesicle trafficking and exocytosis (Rothman and Wieland, 1996). By contrast, unconventional secretion bypasses the ER-Golgi route (La Venuta et al., 2015; Nickel and Rabouille, 2009; Rabouille, 2017; Rabouille et al., 2012; Zhang and Schekman, 2013). Although it only accounts for a small set of secreted proteins, those that use unconventional pathways to exit the cell are of exceptional importance. Both IL-1β and FGF2 exclusively utilise unconventional pathways to exit the cell. Whilst the respective mechanisms for IL-1β and FGF2 have appeared distinct thus far, evidence is now emerging that under some conditions, the mechanism of IL-1β and FGF2 secretion may indeed share some common principles. In this opinion article we discuss the unconventional secretory pathways of FGF2 and IL-1β with a particular emphasis on recent discoveries, suggesting that the well-established role of
phosphatidylinositol 4,5-bisphosphate [PI(4,5)P$_2$]-dependent plasma membrane pore formation in unconventional secretion of FGF2 may also be relevant for IL-1β secretion under certain conditions.

**Processing and unconventional secretion of IL-1β**

IL-1β is mainly produced by cells of the hematopoietic lineage such as macrophages as an inactive precursor, pro-IL-1β, in response to pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). PAMPs are motifs that are expressed by pathogens, such as bacteria and viruses (e.g. bacterial endotoxin), whereas DAMPs are endogenous molecules released from dead or dying cells (e.g. high mobility group box-1 protein, HMGB1), or endogenous molecules that have been modified during disease (e.g. fibrillary amyloid-β (Halle et al., 2008)). PAMPs and DAMPs activate pattern-recognition receptors (PRRs) on cells, which then induce signalling cascades that increase de novo expression of pro-inflammatory molecules such as pro-IL-1β that subsequently accumulates in the cytosol of the cell (Brough and Rothwell, 2007). What happens from this point on depends on cell type and stimulus. There are several recent reviews that cover in detail the mechanisms proposed for the secretion of IL-1β (Daniels and Brough, 2017; Deretic et al., 2012; Lopez-Castejon and Brough, 2011; Piccioli and Rubartelli, 2013; Ponpuak et al., 2015). These include (i) lysosomal and autophagic pathways (Andrei et al., 1999; Zhang et al., 2015), (ii) the shedding of microvesicles (Blanco et al., 2005; MacKenzie et al., 2001), (iii) release via exosomes or other endocytic intermediates (Dupont et al., 2011; Jiang et al., 2013; Kimura et al., 2017a; Kimura et al., 2017b; MacKenzie et al., 2001; Qu et al., 2007; Zhang et al., 2015) and (iv) release by direct translocation across plasma membrane correlating with the subsequent death of the secreting cell (Brough and Rothwell, 2007; Martin-Sanchez et al., 2016; Shirasaki et al., 2014). Despite these different unconventional release mechanisms, the actual pathway remains poorly defined and controversial. In a past review, we proposed that the different mechanisms described in the literature were not mutually exclusive but were engaged as part of a continuum depending
upon the strength and duration of the secretion stimulus (Lopez-Castejon and Brough, 2011). In this Opinion article, we will highlight the most recent developments in the potential secretory route for IL-1β that involve pore formation at the plasma membrane and, in this regard, point to an overlap with the secretory pathway of FGF-2.

LPS-induced stimulation of Toll-like receptor 4 on human monocytes is sufficient to activate the processing machinery (described below) and to allow secretion of mature IL-1β (Gaidt et al., 2016). By contrast, stimulation of macrophages with only PAMPs results in them becoming ‘primed’, and they require a second stimulus to drive the secretion of mature IL-1β (Lopez-Castejon and Brough, 2011). This second stimulus can be either a PAMP, a DAMP, or a signalling molecule or process that induces an alteration in cellular homeostasis (Liston and Masters, 2017), which then activates cytosolic PRRs. The best characterised PRR is the nucleotide-binding domain and leucine-rich repeat containing receptor with a pyrin domain 3 (NLRP3) (Strowig et al., 2012), although, to-date, there are no ligands that have been identified that directly bind and activate NLRP3. Once activated, NLRP3 nucleates the oligomerisation of the adaptor protein apoptosis-associated speck-like protein with a caspase recruitment domain (ASC) to form large multi-molecular complexes, called inflammasomes, which provide the scaffold for the proximity-induced auto-catalytic activation of the protease caspase-1 (Lu et al., 2014). It should be noted however that a number of PRRs in addition to NLRP3 are capable of forming inflammasomes in response to specific pathogen and disease associated signals stimuli (Prochnicki and Latz, 2017). Caspase-1 directly cleaves pro-IL-1β to a mature active form that is then secreted from the cell. In macrophages, a consequence of inflammasome activation is an inflammatory type of cell death called pyroptosis. Pyroptosis is a protective host response that removes the intracellular replicating niche of certain pathogens (Stephenson et al., 2016). In addition, pore-induced intracellular traps, cellular corpses that trap pathogens, are also formed, which facilitate pathogen clearance (Jorgensen et al., 2016). Recently, pyoptotic cell death of
macrophages was found to be dependent upon the pore forming properties of the protein gasdermin D (GSDMD) (Kayagaki et al., 2015; Shi et al., 2015). In addition to the potential secretory pathways outlined above, the formation of plasma membrane pores may now explain the secretion of IL-1β that occurs during pyroptotic cell death. The potential for GSDMD pores as a conduit for IL-1β secretion is further discussed below.

**The unconventional secretory pathway of FGF2**

FGF2 is an extracellular mitogen secreted from a wide range of cell types during development (Beenken and Mohammadi, 2009). Beyond developmental functions of FGF2, for example in angiogenesis, FGF2 plays critical roles under pathophysiological conditions, for instance in tumorigenesis, as primary cancer cells express and secrete large quantities of FGF2 (Akl et al., 2016). In recent years, as described below, we have elucidated the unconventional secretory pathway with regard to both trans-acting factors and cis-elements required for FGF2 membrane translocation (La Venuta et al., 2015). These studies demonstrated that unconventional secretion of FGF2 from cells is mediated by direct translocation across plasma membranes (Nickel, 2011; Schäfer et al., 2004; Steringer et al., 2015) (Fig. 1). Initiated through PI(4,5)P₂-dependent recruitment of FGF2 at the inner leaflet (Nickel, 2011; Temmerman et al., 2008; Temmerman and Nickel, 2009), FGF2 undergoes oligomerization and membrane insertion (Steringer et al., 2012; Steringer et al., 2015). This process depends on two cysteine residues on the molecular surface of FGF2 that form intermolecular disulfide bridges (Müller et al., 2015). Intriguingly, these cysteine residues are absent from all the FGF family members that carry signal peptides for secretion through the ER-Golgi pathway, suggesting that these residues are not required for FGF signalling, but rather have a specific role in unconventional secretion. Consistently, FGF2 variant forms lacking these surface cysteines are not secreted from cells (La Venuta et al., 2015; Müller et al., 2015). Thus, membrane-inserted FGF2 oligomers are the key structural components required for membrane translocation and unconventional secretion of FGF2 from cells (Müller et al., 2015; Nickel, 2011; Steringer et al., 2012; Steringer et al., 2015). The structure
of FGF2 oligomers inserted in the membrane has been proposed to be characterized by a toroidal architecture with the PI(4,5)P₂ binding sites of the central FGF2 oligomer pointing to the periphery of the lipidic membrane pore (Fig. 1) (Steringer et al., 2012; Steringer et al., 2015). This view is supported by the observation that, upon membrane insertion of FGF2 oligomers, both membrane passage of small fluorescent tracers and transbilayer diffusion of membrane lipids can be observed (Steringer et al., 2012; Steringer et al., 2015). Furthermore, diacylglycerol, a cone shaped lipid that interferes with membrane curvature that is stabilized by PI(4,5)P₂, was found to inhibit the insertion of FGF2 oligomers into the membrane (Steringer et al., 2012; Steringer et al., 2015). Based on these findings, we have proposed that the role of PI(4,5)P₂ in the unconventional secretion of FGF2 is three-fold, with (i) recruitment of FGF2 to the plasma membrane, (ii) orienting FGF2 molecules at the inner leaflet to drive oligomerization and (iii) stabilizing local curvature to allow for a toroidal membrane structure surrounding the membrane-inserted FGF2 oligomers.

However, the mechanism by which membrane inserted FGF2 oligomers serve as translocation intermediates is not entirely clear. We recently suggested that dynamic assembly at the inner leaflet and disassembly at the outer leaflet could be a possible mechanism (La Venuta et al., 2015). Disassembly into FGF2 monomers or dimers would be driven by membrane-proximal heparan sulfate proteoglycans on cell surfaces resulting in net transport of FGF2 from the inner leaflet to the outer leaflet of the plasma membrane. Therefore, cell surface heparan sulfates have been termed an extracellular trap for FGF2 that is required for FGF2 translocation into the extracellular space (Nickel, 2007; Nickel, 2011; Nickel and Rabouille, 2009; Nickel and Seedorf, 2008; Zehe et al., 2006). Indeed, FGF2 binds with nanomolar affinity to heparan sulfates, which therefore not only disassemble the membrane-inserted FGF2 oligomer at the outer leaflet, but also retain FGF2 monomers (or dimers) on the cell surface without their release into the cellular supernatant (Engling et al., 2002; Nickel, 2005; Trudel et al., 2000). However, FGF2 has been shown to undergo intercellular spreading by direct cell-cell contacts, which are
probably mediated by direct exchange between heparan sulfate chains that are physically associated with different cell surfaces (Zehe et al., 2006). Thus, during the lifetime of a FGF2 molecule, heparan sulfate proteoglycans have three roles: (i) mediating the final step of FGF2 secretion (Nickel, 2007; Zehe et al., 2006), (ii) protecting FGF2 on cell surfaces against degradation (Nugent and Iozzo, 2000) and (iii) mediating FGF2 signalling as part of a ternary complex comprising FGF2, heparan sulfates and high-affinity FGF receptors (Belov and Mohammadi, 2013; Presta et al., 2005; Ribatti et al., 2007). Based on the sequential interactions of FGF2 with firstly PI(4,5)P₂ at the inner leaflet of the plasma membrane and then the heparan sulfates on cell surfaces, the assembly-disassembly model of FGF2 membrane translocation offers a molecular basis for directionality of FGF2 transport into the extracellular space (La Venuta et al., 2015). This model is consistent with previous studies demonstrating that membrane translocation of FGF2 occurs in a fully folded state (Backhaus et al., 2004; Nickel, 2011; Torrado et al., 2009) as this mechanism requires the formation of defined oligomers with the subunits being properly folded during membrane insertion. In addition, the molecular interactions of FGF2 with both PI(4,5)P₂ and heparan sulfates depend on the proper folding of FGF2 (Torrado et al., 2009). Because FGF2 membrane translocation occurs at the level of the plasma membrane, these findings suggest an intrinsic quality control mechanism that limits unconventional secretion to only fully folded and therefore functional forms of FGF2 (Nickel, 2011; Torrado et al., 2009).

Beyond the core machinery of FGF2 membrane translocation discussed above, two additional trans-acting factors, ATP1A1 (the α-subunit of the Na/K ATPase) and non-receptor tyrosine kinase Tec kinase, have been identified by genome-wide RNAi screening (Ebert et al., 2010; La Venuta et al., 2015; Zacherl et al., 2015). Both ATP1A1 and Tec kinase make direct contacts with FGF2 at the inner leaflet of the plasma membrane (Fig. 1). While the precise role of ATP1A1 is currently unclear, Tec, which has previously been described in the context of immune cell development and activation (Yang et al., 2000), was shown to regulate FGF2 secretion through its tyrosine phosphorylation, which facilitates the
insertion of FGF2 oligomers into the membrane (Steringer et al., 2012; Steringer et al., 2015). Tec kinase contains a pleckstrin homology (PH) domain that mediates its recruitment to the inner leaflet of the plasma membrane through the phosphoinositide PI(3,4,5)P_3. Following the activation of various types of receptors, the levels of PI(3,4,5)P_3 increase, which results in the recruitment of Tec kinase to the inner leaflet of the plasma membrane. Tec kinase then becomes phosphorylated by plasma-membrane resident Src kinases, or by autophosphorylation within its activation loop, resulting in enzymatic activation (Bradshaw, 2010) and subsequent phosphorylation of its targets (Lewis et al., 2001). Therefore, phosphorylation of FGF2 by Tec kinase is likely to occur at the inner leaflet of the plasma membrane (La Venuta et al., 2015; Nickel, 2011). As FGF2 is a key signalling molecule in the context of many cancers, Tec kinase-regulated secretion of FGF2 represents an interesting link with the upregulation of PI3 kinases in many tumour cells (Liu et al., 2009). PI3 kinases catalyse the formation of PI(3,4,5)P_3 and high cellular levels of this phosphoinositide are likely to support efficient secretion of FGF2 which, in turn, enhances tumorigenesis. Of note, small-molecule inhibitors that prevent FGF2 from interacting with Tec kinase have been developed and block both tyrosine phosphorylation and unconventional secretion of FGF2 from cells (La Venuta et al., 2016). These inhibitors are promising lead compounds for the development of drugs aimed at disabling tumour cells to mobilise FGF2 as a survival factor.

In conclusion, the molecular machinery mediating unconventional secretion of FGF2 has been characterized in great detail. It is based upon direct membrane translocation of FGF2 across plasma membranes. At the core of this process, FGF2 is triggered to oligomerize when recruited through PI(4,5)P_2, resulting in membrane insertion of FGF2 oligomers along with the formation of a lipidic membrane pore with a toroidal architecture. These oligomers are disassembled at the outer leaflet of plasma membranes through the action of cell surface heparan sulfates, resulting in directional translocation of FGF2 molecules to cell surfaces.
**Possible PI(4,5)P₂-dependent release of IL-1β**

As mentioned above, caspase-1-dependent pyroptotic cell death requires the processing of GSDMD protein (Kayagaki et al., 2015; Shi et al., 2015). GSDMD is a substrate for inflammatory caspases including caspase-1, and caspase cleavage of full length GSDMD liberates the N-terminal domain (GSDMD-N) from being auto-inhibited by the C-terminal domain (GSDMD-C), allowing it to induce pyroptotic cell death (Shi et al., 2015). As GSDMD cleavage is downstream of caspase-1 activation, it is not required for caspase-1-dependent processing of pro-IL-1β to its mature form. Following canonical NLRP3 inflammasome activation in GSDMD-deficient macrophages, IL-1β secretion is inhibited (He et al., 2015; Shi et al., 2015); however, another study suggests this is only the case following non-canonical inflammasome activation (Kayagaki et al., 2015), also suggesting the possibility of GSDMD-independent IL-1β secretion. Among the phosphoinositides, GSDMD-N specifically binds to PI(4,5)P₂ to form pores that allow for leakage of small fluorescent tracer molecules from PI(4,5)P₂-containing liposomes (Ding et al., 2016). This ability is similar to what has been reported for both FGF2 (Müller et al., 2015; Steringer et al., 2012) and HIV-Tat (Zeitler et al., 2015). GSDMD-N pores can be observed on liposomes using electron microscopy (Ding et al., 2016; Liu et al., 2016) and are suggested to cause pyroptosis through a rapid loss of plasma membrane ionic homeostasis and subsequent osmotic lysis. The size of GSDMD-N-induced pores in liposomes is 10 to 14 nm in diameter, which is sufficiently large to allow the passage of IL-1β (4.5 nm diameter), suggesting that such GSDMD-N pores could potentially serve as the conduit for IL-1β secretion in macrophages (Ding et al., 2016). This is supported by evidence from us, and others, who have reported a precise correlation between a loss of membrane integrity and the extracellular appearance of IL-1β in macrophages (Martin-Sanchez et al., 2016; Shirasaki et al., 2014). Furthermore, we recently reported that neither pro- or mature IL-1β are able to directly bind to PI(4,5)P₂ or permeabilise liposomes, suggesting that they do not form pores such as GSDMD-N, FGF-2 or HIV-Tat (Martin-Sanchez et al., 2016). Thus, although there are numerous pathways for the unconventional secretion of IL-1β and fundamental mechanistic differences exist...
between the mechanisms suggested for IL-1β release and employed by FGF-2, the recent discoveries for GSDMD allow us to speculate the tantalising possibility that like FGF-2, under some conditions, the secretion of IL-1β may occur through PI(4,5)P₂-dependent pores in the plasma membrane (Fig. 2).

**Pore formation as an unconventional mechanism for protein release**

As mentioned above, a consequence of GSDMD-N-dependent pore formation is a rapid pyroptotic cell death (Kayagaki et al., 2015; Shi et al., 2015). However, cell death is not always an inevitable consequence of IL-1β release. Indeed, human monocytes release IL-1β in response to lipopolysaccharide (LPS, a bacterial product used to simulate infection) through a mechanism that involves the NLRP3 inflammasome and caspase-1, but does not result in pyroptosis (Gaidt et al., 2016). Neutrophils also release IL-1β after caspase-1 activation without associated pyroptotic cell death (Karmakar et al., 2015; Karmakar et al., 2016). However, these data do not necessarily suggest that release of IL-1β is independent of GSDMD-N pore formation. We recently reported that the complex polyphenolic compound punicalagin stabilised membranes and prevented pyroptotic release of IL-1β from macrophages, as well as its non-pyroptotic release from neutrophils (Martin-Sanchez et al., 2016), suggesting that there is some commonality between the pathways of IL-1β release despite the different cellular outcome. Furthermore, cells secreting FGF2 do not undergo osmotic lysis (Backhaus et al., 2004; Engling et al., 2002; Florkiewicz et al., 1995; La Venuta et al., 2015; Müller et al., 2015; Temmerman et al., 2008; Torrado et al., 2009; Trudel et al., 2000; Zehe et al., 2006), suggesting that secretory pores can be formed in the absence of cell death. Furthermore, there are some recent reports that highlight GSDMD-independent release of IL-1β and show these occur in the context of other types of cell death. In these instances, inflammasome activation and IL-1β secretion is induced by the mixed lineage kinase domain-like (MLKL) protein (Fig. 2), which is required for the induction of necroptotic cell death (Conos et al., 2017; Gutierrez et al., 2017). Necroptosis, like pyroptosis, is a form
of programmed necrosis and requires disruption of the plasma membrane, which in this case is caused by oligomerisation of MLKL and its insertion into the membrane (Dondelinger et al., 2014). Because binding and membrane permeabilisation of MLKL also requires its binding to PI(4,5)P₂ (Dondelinger et al., 2014), it is also possible that in these cases, the secretion of IL-1β requires PI(4,5)P₂-dependent formation of plasma membrane pores, although this remains speculative (Fig 2).

Conclusions
Although FGF2 and IL-1β are structural homologues (Priestle et al., 1988; Zhu et al., 1991), and are both secreted from cells through unconventional protein release pathways, their respective release mechanisms are distinct. However, recent evidence suggests the potential for some mechanistic overlap. FGF-2 is known to traverse the plasma membrane through PI(4,5)P₂-dependent oligomerization and formation of a lipidic membrane pore with a toroidal architecture (La Venuta et al., 2015; Müller et al., 2015; Steringer et al., 2012; Temmerman et al., 2008). Similarly, HIV-Tat has been shown to be secreted by unconventional means in a PI(4,5)P₂-dependent manner, again involving oligomerization and membrane pore formation (Debaisieux et al., 2012; Rayne et al., 2010; Zeitler et al., 2015). The observations that GSDMD forms pores large enough to allow the passage of IL-1β raises the possibility that under pyroptotic cell death, GSDMD pores could be a conduit allowing IL-1β to reach the extracellular space. However tantalising this concept is, it is not yet fully supported by the literature, and it remains possible that GSDMD supports IL-1β secretion by influencing some additional aspect of the pathway yet to be defined. Thus as methods and tools to study IL-1β secretion become further refined, we can expect to see further insights and clarification of these intriguing mechanisms.

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Figures

Fig. 1: A schematic diagram illustrating the PI(4,5)P_2-dependent formation of FGF2 pores in the plasma membrane and FGF2 secretion (modified from La Venuta et al., 2015). Summarised in the figure is the PI(4,5)P_2-dependent recruitment of FGF2, the resultant membrane insertion of FGF2 oligomers along with the formation of a lipidic membrane pore with a toroidal architecture. The oligomers are disassembled at the outer leaflet of plasma membranes through the action of cell-surface heparan sulfates, resulting in the directional translocation of FGF2 molecules to the cell surface.

Fig. 2: A schematic diagram illustrating the PI(4,5)P_2-dependent formation of GSDMD-N and MLKL pores in the plasma membrane that may facilitate the release of IL-1β into the extracellular space. The toroidal structure with PI(4,5)P_2 head groups bound in the periphery is a hypothesis based upon what is known for FGF2 pores. Upon inflammasome assembly, caspase-1 activates and processes pro-IL-1β and GSDMD. The N-terminal portion of GSDMD binds to PI(4,5)P_2 in the inner leaflet of the plasma membrane, allowing insertion into the lipidic bilayer, thereby forming pores that could potentially allow the direct secretion of IL-1β (dashed lines). Similarly, necoptotic stimuli lead to MLKL phosphorylation, its binding to PI(4,5)P_2 and membrane insertion; this results in pore formation that could also promote IL-1β release from the cell.
References


