# Unconventional secretion: an extracellular trap for export of fibroblast growth factor 2

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## Summary

**Several secretory proteins are released from cells by mechanisms that are distinct from the classical endoplasmic reticulum (ER)/Golgi-mediated secretory pathway. Recent studies unexpectedly revealed that the interaction between one such protein, fibroblast growth factor 2 (FGF-2), and cell surface heparan sulfate proteoglycans (HSPGs) is essential for secretion. FGF-2 mutants that cannot bind to heparan sulfates are not secreted, and cells that do not express functional HSPGs cannot secrete wild-type FGF-2. FGF-2 appears to be secreted by direct translocation across the plasma membrane in an ATP- and membrane-potential-independent manner. I propose that its translocation across the membrane is a diffusion-controlled process in which cell surface HSPGs function as an extracellular molecular trap that drives directional transport of FGF-2.**

Key words: Fibroblast growth factor 2, Membrane translocation, Unconventional protein secretion, Nonclassical export

## Introduction

The majority of extracellular proteins are exported from cells through the classical endoplasmic reticulum (ER)/Golgi-dependent secretory pathway (Lee et al., 2004). They first translocate into the lumen of the ER and then undergo vesicular transport through the Golgi complex to the cell surface. In the case of soluble factors, an N-terminal signal peptide directs secretory proteins into this pathway (Osborne et al., 2005). Therefore, it came as a great surprise when certain soluble extracellular proteins were discovered that do not possess this signal (Cleves, 1997; Hughes, 1999; Nickel, 2003; Prudovsky et al., 2003). The existence of unconventional mechanisms was supported by the demonstration that brefeldin A, a drug that blocks membrane trafficking through the ER/Golgi complex (Lippincott-Schwartz et al., 1989; Misumi et al., 1986), does not affect their secretion rates (Hughes, 1999; Nickel, 2003). Furthermore, unconventionally secreted proteins do not localize to the ER/Golgi complex and consistently lack posttranslational modifications added in these compartments (Hughes, 1999).

Unconventional secretory mechanisms have been revealed for several biomedically important factors, including proangiogenic mediators such as fibroblast growth factor 2 (FGF-2), inflammatory cytokines such as interleukin 1α and 1β as well as regulators of cell fate belonging to the galectin family (Nickel, 2003; Prudovsky et al., 2003). Diverse mechanisms have been proposed to explain these unconventional secretory processes, including lysosomal secretion, plasma membrane shedding, release in exosomes as well as secretion through transporters that reside in the plasma membrane (Nickel, 2005), and multiple mechanisms have even been proposed for individual unconventionally secreted proteins. For example, interleukin 1β has been reported to be released both by lysosomal secretion (Andrei et al., 1999; Andrei et al., 2004) and by plasma membrane shedding (MacKenzie et al., 2001). Here, I discuss a new model for the molecular mechanism of FGF-2 secretion based on recent data. Key aspects of this model are: (1) direct translocation of FGF-2 from the cytoplasm across the plasma membrane in the absence of transport vesicles; (2) the independence of membrane translocation from ATP hydrolysis or a membrane potential; (3) diffusion-controlled membrane translocation process; and (4) an extracellular molecular trap formed by membrane-proximal heparan sulfates that ensures directional transport of FGF-2 into the extracellular space.

## Passive versus active mechanisms of translocation

Central to the molecular mechanism of FGF-2 secretion is the question of what actually drives translocation of FGF-2 across the plasma membrane in terms of energy requirements. Studies employing an intact cell model system led to the proposal that the overall process of FGF-2 secretion in living cells depends on ATP hydrolysis (Florkiewicz et al., 1995). However, the early data did not really demonstrate that membrane translocation itself is driven by ATP hydrolysis. Depletion of ATP from intact cells affects many fundamental cellular functions and, therefore, the inefficient FGF-2 secretion observed could well be an indirect effect. An in vitro approach using plasma-membrane-derived inside-out vesicles shows that FGF-2 can translocate directly across the plasma membrane but that neither ATP hydrolysis nor a membrane potential is required (Schäfer et al., 2004). FGF-2 thus appears to traverse the plasma membrane by passive diffusion.

At first glance, passive diffusion seems unusual compared with other membrane translocation processes, such as import into the mitochondrial matrix (driven by ATP hydrolysis) (Neupert and Herrmann, 2007) or the bacterical twin arginine secretion system (driven by a membrane potential) (Lee et al.,

2006). So are there other examples in which proteins traverse a membrane by diffusion? Indeed, this is the case for the posttranslational translocation of secretory proteins across the ER membrane, a process mediated by the Sec61 complex (Osborne et al., 2005). The molecular chaperone BIP is required at the luminal side of the ER to promote directional protein translocation across the membrane. Although BIP hydrolyzes ATP, the nucleotide binding and hydrolysis cycle regulates its association with and dissociation from its substrates. Thus, BIP-mediated ATP hydrolysis is important to promote multiple rounds of action rather than actively drive translocation across the membrane (Panzner et al., 1995).

Intriguingly, membrane translocation of ER proteins can occur in the absence of BIP and ATP in vitro on Sec61-bearing proteoliposomes whose lumens contain antibodies directed against the translocated substrate (Matlack et al., 1999). Under these conditions, the translocation process is rendered ATP-independent and is driven by a molecular trap formed by the antibodies. Thus, membrane translocation is mediated by passive diffusion and, therefore, occurs in both directions (Liebermeister et al., 2001). However, net directional transport is achieved by binding of the substrate to BIP at the luminal side of the liposomal membrane. Thus, BIP acts as a molecular ratchet preventing backward diffusion of translocation substrates; Brownian motion is thus the basis of posttranslational translocation across the ER membrane (Matlack et al., 1999).

Another example of ATP-independent translocation across a membrane is the import of small proteins into the intermembrane space (IMS) of mitochondria (Herrmann and Hell, 2005). These proteins are transported across the outer membrane of mitochondria by passive diffusion in a loosely folded conformation, a process that is mediated by a proteinaceous pore generated by the TOM complex (Chacinska et al., 2004). Directional transport of small IMS proteins such as TIM9 and TIM10 depends on conserved patterns of cysteine residues (Mesecke et al., 2005). These are crucial for folding reactions in the intermembrane space that involve a disulfide-relay system based on the proteins Mia40 and Erv1 (Chacinska et al., 2004; Mesecke et al., 2005; Naoe et al., 2004). TIM proteins thus get trapped in the intermembrane space by a folding mechanism that prevents them from diffusing back into the cytoplasm. Irrespective of the trapping mechanism, in both examples the translocation step is not driven by ATP hydrolysis but instead occurs by passive diffusion. FGF-2 translocation might therefore function in a similar manner because it is not driven by ATP hydrolysis or a membrane potential.

## An extracellular molecular trap ensures net directional transport of FGF-2 into the extracellular space

Because FGF-2 translocation is a diffusion-controlled process (Schäfer et al., 2004), some mechanism must promote net transport of FGF-2 from the cytoplasm into the extracellular space. Recent findings suggest that a molecular trap plays a central role in FGF-2 secretion (Zehe et al., 2006) (Fig. 1). Extracellular FGF-2 forms a ternary complex with heparan sulfate proteoglycans (HSPGs) and high-affinity FGF receptors (Pellegrini et al., 2000; Schlessinger et al., 2000). FGF receptors, however, are not essential for FGF-2 secretion because Chinese hamster ovary (CHO) cells generally do not express them (Rusnati et al., 2002) and yet secrete FGF-2 (Engling et al., 2002; Backhaus et al., 2004). CHO cells do express HSPGs, and these molecules probably represent the principal FGF-2-binding sites on the surface of mammalian cells. The heparan-sulfate-binding site in FGF-2 is contained in the C-terminal part. Two lysine-rich surface loops have been implicated in specific binding to HSPGs, which display nanomolar affinity (Faham et al., 1996; Faham et al., 1998; Raman et al., 2003).

Intriguingly, mutant forms of FGF-2 that cannot bind to HSPGs as a result of C-terminal truncations are not released from cells. Similarly, wild-type FGF-2 is not secreted from cells whose HSPGs are compromised (Zehe et al., 2006) either as a consequence of somatic mutations (Esko, 1991; Esko et al., 1985) or through treatment with chlorate, which blocks sulfation of the sugar side chains of HSPGs (Baeuerle and Huttner, 1986; Conrad, 2001; Safaiyan et al., 1999).

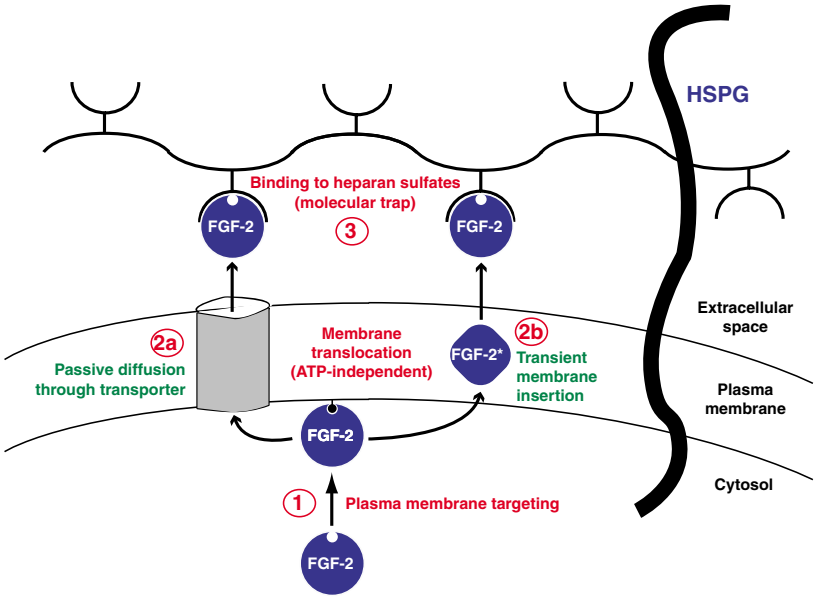


**Fig. 1.** Schematic model for FGF-2 secretion. Nonclassical FGF-2 export from mammalian cells can be dissected into three steps. (1) Plasma membrane targeting; (2) membrane translocation; and (3) extracellular trapping by binding to HSPGs. FGF-2 membrane translocation does not depend on ATP hydrolysis or a membrane potential but rather is a diffusion-controlled process. Net transport to the extracellular space is established by a molecular trap consisting of HSPGs, the heparan sulfate binding sites for FGF-2 being located close to the extracellular membrane surface.

Reconstitution experiments provide further evidence for a direct requirement for extracellular HSPGs in FGF-2 export. When HSPG-deficient cells expressing FGF-2 are grown together with wild-type CHO cells that express HSPGs but lack FGF-2, secretion of FGF-2 from the HSPG-deficient cells is restored, which presumably indicates that HSPGs can act in trans. Intriguingly, export of FGF-2 from HSPG-deficient cells is only observed when HSPGs expressed in trans are close to the surface of FGF-2-expressing HSPG-deficient cells (Zehe et al., 2006).

Interaction between FGF-2 and HSPGs is thus clearly essential for the FGF-2 export process, and, on the basis of the experimental observations described above, the following hypotheses can be developed. The molecular trap might simply work as an extracellular sink that maintains a steep concentration gradient between the cytoplasmic pool and the soluble extracellular population of FGF-2. Under steady-state conditions, however, only approximately 10% of FGF-2 molecules are associated with the cell surface; the remaining 90% reside in the cytoplasm (Engling et al., 2002; Zehe et al., 2006). In addition, extracellular HSPGs need to be available close to the FGF-2 translocation sites in plasma membranes (Zehe et al., 2006). These observations are consistent with a more active role of HSPGs. This might be to extract FGF-2 molecules from the plasma membrane as the final step of the FGF-2 secretion process. Thus, in addition to a steep concentration gradient of FGF-2 between cytoplasmic and extracellular pools of soluble FGF-2, the binding energy that is made available when FGF-2 binds to extracellular HSPGs may facilitate net transport of FGF-2 into the extracellular space.

## A general export mechanism for unconventional secretory lectins?

How far does all this apply to other unconventional secretory processes? In common with FGF-2, several unconventional secretory proteins are lectins. Members of the galectin family, for example, are β-galactoside-specific lectins of the extracellular matrix (Liu and Rabinovich, 2005) and use an unconventional export mechanism (Hughes, 1999; Nickel, 2003). Binding to cell surface glycoproteins or glycolipids containing β-galactosides such as the glycolipid $GM_1$ (Kopitz et al., 1998) has been shown to be crucial for secretion of galectin 1 (Seelenmeyer et al., 2005). Similarly, unconventional secretion of CGL-2, a distant relative of mammalian galectin 1 from the multicellular fungus *Coprinopsis cinerea*, depends on the presence of a functional β-galactoside-binding site in CGL-2 (Seelenmeyer et al., 2005). Because galectin-1 and CGL-2 share only very limited sequence similarity, the targeting signals for export of β-galactoside-specific lectins are probably not based on sequence elements but rather on determinants in the three-dimensional structure, such as the β-galactoside-binding site. Indeed, the similarity between galectin-1 and CGL-2 was only discovered when the atomic structures of both proteins had been solved and the typical galectin fold was found to be present in CGL-2 (Walser et al., 2004).

These observations may point to a more broadly applicable mechanism of unconventional secretion of lectins in which an extracellular molecular trap drives the export process. However, it remains to be shown whether this idea can really be extended to other closely related lectins secreted by unconventional means. For example, in the case of FGF-1, a close relative of FGF-2, whether cell surface HSPGs play a role in the overall process has so far not been analyzed. FGF-1 secretion is a regulated process, induced by stresses such as heat-shock treatment (Prudovsky et al., 2003), and it is possible that the two export routes differ in multiple aspects.

## Quality control during FGF-2 secretion

Besides providing the basis for an ATP-independent molecular trap model, the recent work has further implications for the mechanism of FGF-2 and galectin-1 secretion. Both FGF-2 and galectin-1 must appear at the cell surface at least in a partially folded state because the proposed trapping mechanism is based on the recognition of their sugar-binding sites (Seelenmeyer et al., 2005; Zehe et al., 2006). This would be consistent with earlier findings demonstrating that FGF-2 does not need to be unfolded for secretion to occur (Backhaus et al., 2004). This observation adds another interesting aspect to the secretory mechanisms of FGF-2 and galectin-1: folding of FGF-2 and galectin-1 might not only be compatible with membrane translocation but in fact be a requirement. If this were true, it might reflect a quality control mechanism at the plasma membrane that ensures the secretion only of properly folded and, therefore, functional FGF-2 and galectin-1 molecules.

## FGF-2 targeting and membrane translocation – a speculative hypothesis

The biggest challenge for future studies in the field is clearly to unravel the mechanisms by which FGF-2 is targeted to the plasma membrane and the structural aspects of its membrane translocation. As depicted in Fig. 1 (step 1), targeting to the plasma membrane can be considered the initial step of FGF-2 secretion. One attractive idea for how a transient interaction of FGF-2 with plasma membranes could be achieved is its recruitment by specific phosphoinositides. Several proteins associated with the inner leaflet of plasma membranes have been shown to interact with phosphatidyl-4,5-bisphosphate [$PI(4,5)P_2$; (Behnia and Munro, 2005)], which is highly enriched in the inner leaflet of plasma membranes but is present only in small quantities in other subcellular membranes (Di Paolo and De Camilli, 2006; McLaughlin et al., 2002). At least in some cases, such interactions are mediated by clusters of basic residues (Heo et al., 2006), which are indeed present in the C-terminal part of FGF-2. In addition, a phosphate ion has been co-crystallized with recombinant FGF-2, coordinated by residues Asn35, Arg128 and Lys133 (Kastrup et al., 1997). Thus, it does not seem too far-fetched that FGF-2 might be targeted to plasma membranes in this way; however, under steady-state conditions perceivable amounts of FGF-2 are not localized to plasma membranes. Therefore, the interaction with phosphoinositides at the plasma membrane would have to be very transient in preparation for the membrane translocation process.

This brings us to the next obvious question: how could membrane targeting and translocation be linked? Two scenarios seem possible. A proteinaceous membrane transporter that has a hydrophilic channel may exist (Fig. 1, step 2a). Such a pore might allow membrane translocation by passive diffusion and could work in conjunction with both phosphoinositides at the inner leaflet and the HSPG-mediated molecular trap on the extracellular side. Note that

phosphoinositides such as PI(4,5)$P_2$ are enriched in lipid microdomains (McLaughlin et al., 2002) and, therefore, might potentially be able to locally concentrate FGF-2 at sites close to the putative transporter. If FGF-2 is indeed transported in a fully folded state, such a pore might be similar to that in the twin arginine secretion system from bacteria (Lee et al., 2006); however, the driving force would be different. As in the case of protein translocation mediated by the twin arginine system, at this point it is unclear how such a transporter could accommodate FGF-2 in its fully folded state.

Alternatively, an as-yet-unrecognized property of FGF-2 might enable its passage through the membrane by a transporter-independent mechanism (Fig. 1, step 2b). Again, it is tempting to think of phosphoinositides because interactions between proteins and PI(4,5)$P_2$ can result in a conformational change (Milburn et al., 2003). As depicted in Fig. 1, upon a potential interaction with PI(4,5)$P_2$, FGF-2 might acquire a new conformation (FGF-2*) that stimulates both release from PI(4,5)$P_2$ and membrane insertion (Fig. 1, step 2b). This hypothesis is attractive because it would explain why HSPGs are essential for FGF-2 secretion: they would be needed to extract FGF-2* from the membrane on the extracellular side (Fig. 1, step 3). The binding to HSPGs could cause folding of FGF-2 back to its water-soluble form. This model would also be consistent with the experimental finding that HSPGs are required for FGF-2 secretion in a membrane-proximal orientation (Zehe et al., 2006).

So far no convincing experimental evidence has been reported for either of these two possible mechanisms of membrane translocation. However, FGF-1, a close relative of FGF-2, has been proposed to be able to acquire a so-called molten globule conformation that might allow the protein to penetrate membranes (Prudovsky et al., 2003). Interestingly, FGF-1 has been suggested to destabilize membranes through its ability to interact with acidic membrane lipids (Graziani et al., 2006). Biochemical studies using chemically defined liposomes to reconstitute FGF-2 membrane translocation in vitro as well as genome-wide RNAi screening procedures to identify gene products (e.g. a potential membrane transporter) involved in FGF-2 secretion will be key approaches in our attempts to elucidate the molecular mechanism of FGF-2 secretion further.

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