# Unconventional Mechanisms of Protein Transport to the Cell Surface of Eukaryotic Cells

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## Key Words

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## Abstract

The classical secretion of soluble proteins and transport of integral membrane proteins to the cell surface require transit into and through the endoplasmic reticulum and the Golgi apparatus. Signal peptides or transmembrane domains target proteins for translocation into the lumen or insertion into the membrane of the endoplasmic reticulum, respectively. Here we discuss two mechanisms of unconventional protein targeting to plasma membranes, i.e., transport processes that are active in the absence of a functional Golgi system. We first focus on integral membrane proteins that are inserted into the endoplasmic reticulum but that, however, are transported to plasma membranes in a Golgi-independent manner. We then discuss soluble secretory proteins that are secreted from cells without any involvement of the endoplasmic reticulum and the Golgi apparatus.

## Contents



# GOLGI-INDEPENDENT TRANSPORT OF MEMBRANE PROTEINS TO THE PLASMA MEMBRANE

## Distinct Pathways Out of the ER

Transport along the classical secretory pathway is mediated by vesicular carriers, which recruit cargo, bud off a donor membrane, and fuse with a specific target membrane. Coated vesicles (COPII and COPI) transport the cargo between the endoplasmic reticulum (ER) and the Golgi apparatus (Lee et al. 2004). The sorting of proteins begins to occur as COPII vesicles exit the ER and continues in the Golgi network, allowing for the delivery of cargo to various places, including the plasma membrane (Griffiths & Simons 1986). There are a few examples of proteins that leave the ER in the absence of functioning COPII coat components. Secretion of Hsp150 in the yeast *Saccharomyces cerevisiae* occurs normally in the presence of defective COPII coat proteins Sec24 and Sec13 (Fatal et al. 2002, Karhinen et al. 2005). Reduced activity of Sar1 GTPase fails to abolish the trafficking of voltage-gated $K^+$ channel (Kv4) and its regulator KChlP1 in dendritic outposts of neurons (Hasdemir et al. 2005). Transport of proteins directly to the plasma membrane from the ER may also occur during phagocytosis in dendritic cells of the mouse immune system (Gagnon et al. 2002). Desjardins and collegues speculated that a SNARE-mediated direct fusion of ER-derived vesicles with the plasma membrane allows rapid forward transport without passage through the Golgi (Gagnon et al. 2002). This was based on the in vitro observation of a fusogenic SNARE pair consisting of the ER- and Golgi-localized Sec22 and the plasma membrane–localized Sec9 proteins (McNew et al. 2000). However, the existence of this pathway remains controversial (Becker et al. 2005, Touret et al. 2005, Rogers & Foster 2007).

Researchers have reported various lines of evidence for Golgi-independent transport of membrane proteins to cell surfaces. During passage through the Golgi, N-linked glycan chains are modified so that glycoproteins at the cell surface are endoglycosidase H (endo H) resistant. However, an endo H–sensitive form of the receptor protein-tyrosine phosphatase CD45 rapidly accumulates at the cell surface in T lymphoma cells (Baldwin & Ostergaard 2001, 2002). A similar phenomenon has been observed for the trafficking of the cell adhesion molecules caspr/paranodin and F3/contactin in neuroblastoma N2a cells. These proteins are recruited into lipid microdomains and reach the interface of axonal membranes and myelinating glial cells as endo H–sensitive glycoproteins as

well (Bonnon et al. 2003). Moreover, the rapid transport of CD45 and cell adhesion molecules is brefeldin A (BFA) resistant (Baldwin & Ostergaard 2002, Bonnon et al. 2003). BFA is a fungal metabolite that inhibits the activation of ADP-ribosylation factor 1 (ARF1) on Golgi membranes and, therefore, blocks vesicular transport along the secretory pathway (Misumi et al. 1986, Lippincott-Schwartz et al. 1989, Orci et al. 1991). Golgi-independent trafficking occurs only in specific cell types, indicating that it is a regulated process limited to specific physiological conditions that can operate in addition to trafficking along the classical secretory pathway (Baldwin & Ostergaard 2002, Bonnon et al. 2003).

## Trafficking of Ist2 Defines a Novel Pathway for Direct Transport from the ER to the Plasma Membrane

Although the molecular function of the polytopic membrane protein Ist2 remains elusive, it has been observed that *ist2*Δ mutants showed an increased sodium tolerance, which led to the name Ist2 (Entian et al. 1999). Ist2 belongs to a group of proteins that are translated from localized mRNAs (Long et al. 1997; Takizawa et al. 1997, 2000; Shepard et al. 2003; Aronov et al. 2007). Localized mRNAs are transported along a polarized actin cytoskeleton into the growing daughter cells (buds) of *S. cerevisiae* (summarized by Gonsalvez et al. 2005, Müller et al. 2007). The coordinated transport of She2-bound mRNAs and tubular ER leads to the translation of mRNAs for membrane proteins at the cortical ER in the bud (Schmid et al. 2006, Aronov et al. 2007). The transport of Ist2 to the periphery of mother and daughter cells occurs independently of trafficking through the ER/Golgi system, as shown by the localization of Ist2 in conditional yeast mutants defective in COPII vesicle formation (*sec23-1* and *sec12-4* mutants), intra-Golgi transport (*sec7-1* mutant), and general transport vesicle fusion (*sec18-1* mutant) (Juschke et al. 2004, 2005). *SEC18* encodes the ATPase N-ethylmaleimide-sensitive fusion (NSF) protein. All SNARE-mediated vesicular transport ceases rapidly after a shift to the nonpermissive temperature in *sec18-1* mutants (Graham & Emr 1991). This suggests that the transport of Ist2 occurs without or at low activity of SNARE-mediated membrane fusion. The detection of newly synthesized Ist2 at peripheral patches by fluorescence microscopy, and the degradation of the majority of Ist2 (50–100%) by externally added proteases in intact *Sec* mutant cells, demonstrates that Ist2 can reach specific domains of the cell surface without ER-to-Golgi transport (Juschke et al. 2004, 2005). Some of the Ist2 may remain at specific sites of the cortical ER because the Ist2 patches colocalize with certain cortical ER proteins (Juschke et al. 2004).

Experiments with a chimera of Prm1, a pheromone-induced membrane protein, and the C-terminal domain of Ist2 show that the C terminus is sufficient for Ist2 sorting (Juschke et al. 2005). On its own, Prm1 accumulates at contact sites between cells of opposite mating types before being endocytosed (Heiman & Walter 2000). Tagging with the Ist2 C terminus efficiently relocates the chimera into stable peripheral sites, demonstrating that the Ist2 C terminus contains a dominant sorting signal (Juschke et al. 2005). Although the Prm1-Ist2 chimera receives complete N-linked glycosylation, indicating passage through the ER, only a minor fraction is modified in the *cis*-Golgi. Because this modification depends on the function of Sec18/NSF, one can assume that a small amount of Ist2 enters the classical secretory pathway. None of the Prm1-Ist2 chimera are further modified at the medial- or the *trans*-Golgi. On the basis of these results and the presence of a di-lysine ER-retrieval signal at the extreme C terminus of Ist2, it is likely that Ist2 returns from the *cis*-Golgi to the ER, instead of traveling through the entire Golgi. Also, the Ist2 sorting signal redirects a Gef1-Ist2 chimera from *trans*-Golgi network/endosomes to the cell periphery and circumvents cleavage of Gef1 by the Golgi protease Kex2 (Juschke et al. 2005).

Is the local translation of Ist2 at the cortical ER a prerequisite for unconventional

transport to the neighboring plasma membrane, or does Ist2 trafficking operate independently of mRNA localization? The direct coupling of local translation and trafficking may occur at contact sites between the cortical ER and the plasma membrane. At these sites, the minimal distance between the two membranes is less than 10 nm (Pichler et al. 2001), which approximates the size of a single protein. There is evidence that such narrow cytoplasmic gaps are involved in nonvesicular exchange of lipids (Baumann et al. 2005, Raychaudhuri et al. 2006). However, experiments in mutants without mRNA transport and disruption of the *IST2* mRNA localization element reveal that a coupling of local translation and trafficking is unlikely (Takizawa et al. 2000, Juschke et al. 2004, Franz et al. 2007). Instead of an essential role in targeting, *IST2* mRNA localization operates upstream and separately from a post-translational sorting mechanism. The localization of mRNA ensures that buds express Ist2 locally but has no effect on the sorting of Ist2 to the cell periphery per se.

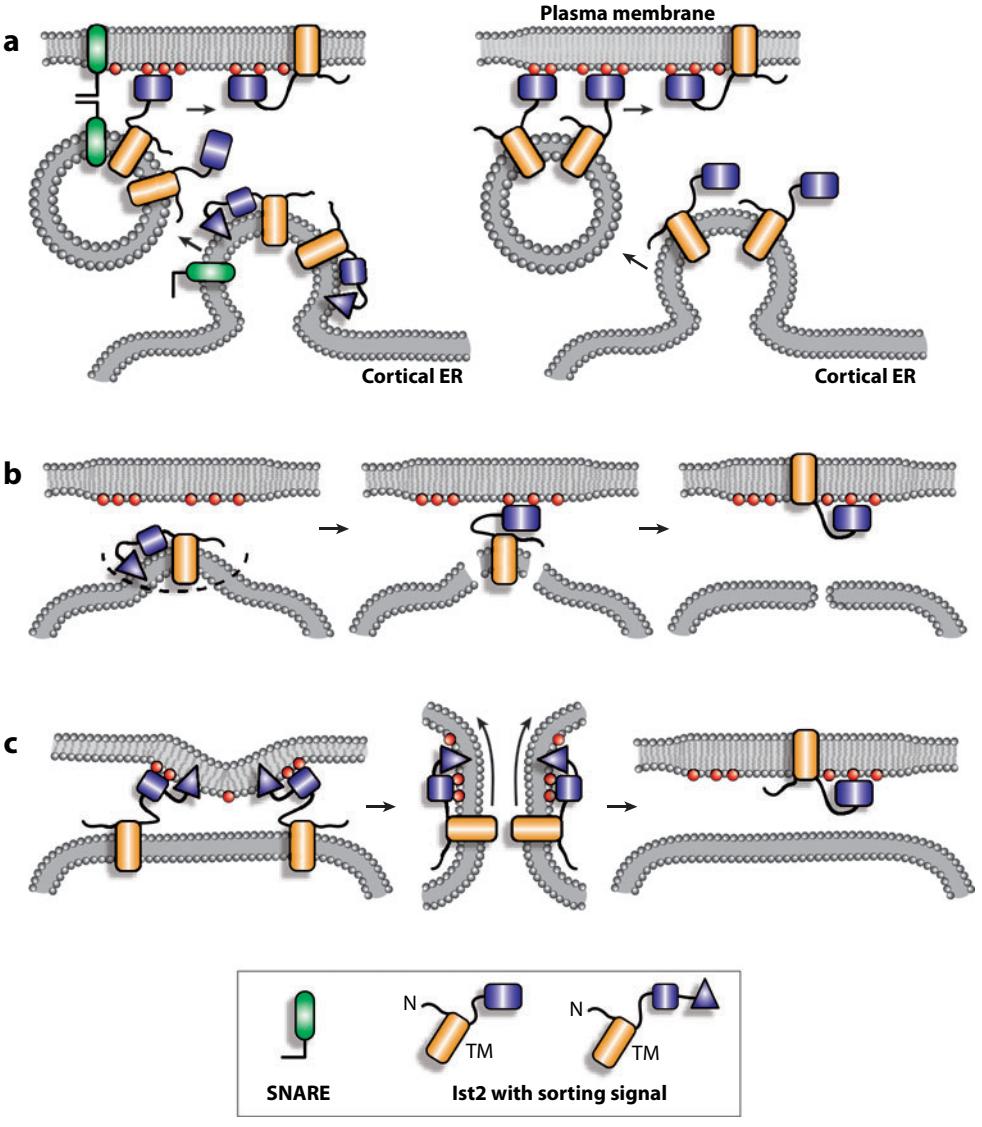
The sorting of Ist2 can be further divided in two distinct steps. First, the efficient accumulation of Ist2 at the cortical ER of mother and daughter cells depends on a protein-sorting signal at the C terminus. A C-terminal truncation of the last 18 residues trapped Ist2 in dot-like structures at the perinuclear ER and cortical ER (Franz et al. 2007). The existence of transport from the general ER to domains of the cortical ER is supported by the observation that wild-type cells, with functioning *IST2* mRNA transport, synthesize small amounts of Ist2 protein at the perinuclear ER (K. Maass, M.A. Fischer, M. Seiler, K. Temmerman, W. Nickel & M. Seedorf, submitted manuscript). Ist2 has not been detected in the perinuclear ER at steady state because it is rapidly transported to the cortical ER via ER tubules (Takizawa et al. 2000; Juschke et al. 2004, 2005; Franz et al. 2007). This rapid transport to the cortical ER depends on two basic clusters at the Ist2 C terminus (K. Maass, M.A. Fischer, M. Seiler, K. Temmerman, W. Nickel & M. Seedorf, submitted manuscript). Each cluster contains four lysines. Owing to the proximity of the cortical ER and the plasma membrane, these basic clusters may bind negatively charged derivates of phosphoinositides (PIPs) at the plasma membrane (McLaughlin et al. 2002), leading to a recruitment of Ist2 at specific sites of the cortical ER. Compared with uncharged or single negatively charged lipids, phosphatidylinositol 4,5 bisphosphate [$PI(4,5)P_2$] has a valence of –4 at pH 7.0 (McLaughlin et al. 2002). Therefore, a protein with a cluster of four or more basic residues on its surface could be sequestered by a $PI(4,5)P_2$-containing membrane.

**Figure 1**

Three different models for the transfer of Ist2 from specific domains of the cortical endoplasmic reticulum (ER) to the plasma membrane. (*a*) The Ist2 sorting signal recruits Ist2 into vesicles that bud off the cortical ER, and these vesicles fuse SNARE-dependently (*left*) or SNARE-independently (*right*) with the plasma membrane. The penetration of the amphipathic α-helix of the Ist2 sorting signal into the cortical ER promotes vesicle budding. Instead of a SNARE-mediated fusion, the activity of the Ist2 sorting signal alone mediates the fusion of vesicles from the cortical ER with the plasma membrane. (*b*) The interaction between the Ist2 sorting signal and cortical ER destabilizes the lipid bilayer and allows the transition of lipid-embedded Ist2 from the ER into the plasma membrane. (*c*) The Ist2 sorting signal binds to phosphatidylinositol 4,5 bisphosphate [$PI(4,5)P_2$] (*red dots*) at the plasma membrane, which triggers the formation of the amphipathic α-helix. The insertion of multiple amphipathic α-helices into the cytosolic leaflet of the plasma membrane induces bending of this membrane domain toward the cortical ER, resulting in transient formation of a fusion pore. This allows diffusion of Ist2 followed by disassembly of the pore and separation of cortical ER and plasma membrane. (*Inset*) Schematic representations of a SNARE protein (in *green*, *left*) and Ist2 [membrane-spanning part with eight transmembrane (TM) domains in *yellow*] with two conformations of the sorting signal (in *blue*). The triangle indicates the transition of the sorting signal into an amphipathic α-helical fold.

## Models for the Direct Transfer of Ist2 from the ER to the Plasma Membrane

Three models may explain the transfer of Ist2 from specific sites of the cortical ER to the plasma membrane. The first model is based on a mechanism involving the recruitment of Ist2 into vesicles that bud from domains of the cortical ER and fuse with the neighboring plasma membrane (**Figure 1*a***). Efficient transport of Ist2 requires the last 69 amino acid residues. When fused to the C terminus of a membrane protein, this sequence was sufficient for transport to the cell periphery (Franz et al. 2007). A multimerization domain can replace the activity of the N-terminal part of the sorting signal (Franz et al. 2007), suggesting that the Ist2 sorting signal functions as multimer. The C-terminal part contains two basic clusters, which partially overlap with a short amphipathic α-helix, and a consensus sequence of a di-lysine ER retrieval signal, which is part of the most C-terminally located basic cluster



KLKKKL (K. Maass, M.A. Fischer, M. Seiler, K. Temmerman, W. Nickel & M. Seedorf, submitted manuscript). Amphipathic α-helices have the potential to penetrate into one leaflet of a membrane, and this penetration can result in local membrane curvature (McMahon & Gallop 2005, Zimmerberg & Kozlov 2006). If the Ist2 sorting signal interacts with the cortical ER, penetration of the amphipathic α-helix may promote vesicle budding, which may be assisted by the recruitment of a COPI coat via the di-lysine ER retrieval signal. In vitro experiments have shown that the COPI coat can bud vesicles directly from the ER (Bednarek et al. 1995). Following budding from the cortical ER, these vesicles may fuse with the plasma membrane, and the ER-located v-SNARE Sec22 and the plasma membrane t-SNARE Sec9 are candidates to mediate this type of membrane fusion (McNew et al. 2000). However, normal trafficking of Ist2 to the cell periphery in *sec18-1* (Juschke et al. 2005) and *sec22*Δ (C. Juschke & M. Seedorf, unpublished observation) mutants argues against SNARE-mediated fusion between the ER and the plasma membrane. Perhaps the Ist2 sorting signal itself can mediate vesicle fusion. The specific environment of the plasma membrane may induce a conformational change of the Ist2 sorting signal so that the signal can switch between states interacting with either the cortical ER or the plasma membrane. An interaction between the positive residues of the Ist2 sorting signal with specific lipids at the plasma membrane may bring Ist2-containing vesicles and the plasma membrane in close proximity (**Figure 1*a***, right). Certain lipids such as PI(4,5)$P_2$ can be highly concentrated on the cytoplasmic side of the plasma membrane and therefore are good candidates as binding sites of the Ist2 sorting signal (McLaughlin et al. 2002). Whether such interactions may lead to membrane fusion remains unclear.

In a second, nonvesicular mechanism, the Ist2 sorting signal could target Ist2 to a specific microenvironment of the interface between the cortical ER and the plasma membrane. Lipid-embedded Ist2 could be extracted from the ER by a mechanism similar to the ER dislocation of class I major histocompatibility complex (MHC) molecules (Ploegh 2007) followed by pore closure of the cortical ER and the reinsertion of Ist2 into the proximal plasma membrane (**Figure 1*b***). This model requires the formation of unstable nonbilayer cortical ER domains containing Ist2. Again, a candidate lipid for this membrane destabilization is PI(4,5)$P_2$, which may be synthesized by the plasma membrane–located lipid kinases Stt4 and Mss4 at cortical ER plasma membrane contact sites (Strahl & Thorner 2007).

A third nonvesicular mechanism could be based on the penetration of the amphipathic α-helix into specific domains of the plasma membrane. At a high local concentration of Ist2, penetration could lead to a bending of the plasma membrane toward the cortical ER, which could cause a fusion pore to form (**Figure 1*c***). Such a pore would allow diffusion of Ist2 from the cortical ER to the plasma membrane. To avoid a substantial release of luminal ER content, the postulated fusion pores must be tightly regulated and transient. The described increased sodium tolerance of *ist2*Δ mutants is consistent with a fusion pore model (Entian et al. 1999).

Taken together, in addition to transport along the classical secretory pathway, eukaryotic cells employ an alternative transport route for fast and regulated transport of specific membrane proteins to specific regions of the plasma membrane. The future goal will be to identify the lipid and protein factors required for the function of this pathway. For that task, trafficking of Ist2 in yeast is an excellent model, and it will be interesting to see which steps of this pathway are conserved in mammalian cells.

## UNCONVENTIONAL SECRETION OF SOLUBLE PROTEINS FROM EUKARYOTIC CELLS

### History of the Discovery of Unconventional Secretory Processes

Interleukin 1β (Rubartelli et al. 1990) and galectin-1 (Cooper & Barondes 1990) were first

demonstrated to be secreted by unconventional means almost 20 years ago. These proteins are not unusual exceptions, but rather these findings led to the identification of additional examples of secretory proteins that escape cells by unconventional means. Initially, it was thought that cells unspecifically released unconventional secretory proteins following exposure to either stress or mechanical wounding (McNeil et al. 1989), as occurs during inflammation and angiogenesis. However, further studies clearly demonstrated that unconventional protein secretion is not paralleled by the release of unrelated cytoplasmic proteins and, in many cases, represents a temperature-sensitive and energy-consuming process (Cleves 1997, Hughes 1999, Nickel 2003). What are the criteria being used to define unconventional secretory proteins? Originally, on the basis of primary structure, factors known to act in the extracellular space, such as interleukin 1β, were recognized as leaderless secretory proteins because they lacked classical signal peptides (Muesch et al. 1990, Rubartelli et al. 1990). Furthermore, BFA, a drug that blocks the ER/Golgi-dependent secretory pathway, does not inhibit secretion of unconventional secretory proteins (Cleves 1997, Hughes 1999, Nickel 2003, Prudovsky et al. 2003). Consistent with these observations, unconventional secretory proteins do not localize to the ER/Golgi system and do not contain posttranslational modifications specific for these compartments (Hughes 1999, Nickel 2003).

## An Overview of Extracellular Proteins Secreted by Unconventional Means

Many of the known unconventional secretory proteins are cytokines, growth factors, or other molecules with important signaling roles in physiological processes such as inflammation, angiogenesis, cell differentiation, or proliferation. In most cases, unconventional protein secretion is a regulated process that is induced by external triggers. Also, unconventional secretory proteins can be grouped into two subclasses. The first class can be defined by factors that function primarily in the extracellular space and are represented by interleukin 1α (Siders et al. 1993, Watanabe & Kobayashi 1994, Tarantini et al. 2001) and interleukin 1β (Rubartelli et al. 1990, Hamon et al. 1997, Zhou et al. 2002). Various cell types release interleukin 1α following heat shock treatment, whereas monocytes and macrophages export interleukin 1β in response to bacterial lipopolysaccharides (LPS) and extracellular ATP. Other examples of unconventional secretory proteins with extracellular functions are galectin-1 (Cooper & Barondes 1990, Cho & Cummings 1995, Cleves et al. 1996, Hughes 1999, Seelenmeyer et al. 2005) and galectin-3 (Sato et al. 1993; Sato & Hughes 1994; Mehul & Hughes 1997, 1999; Thery et al. 2001; Zhu & Ochieng 2001), as well as fibroblast growth factor-1 (FGF-1) (Jackson et al. 1992, 1995; Carreira et al. 1998; LaVallee et al. 1998; Landriscina et al. 2001a,b; Prudovsky et al. 2002) and fibroblast growth factor-2 (FGF-2) (Mignatti & Rifkin 1991, Mignatti et al. 1992, Florkiewicz et al. 1995, Trudel et al. 2000, Engling et al. 2002, Backhaus et al. 2004, Schäfer et al. 2004, Zehe et al. 2006). Galectin-1, a cell-fate regulator with multiple signaling functions in cell differentiation and related processes (Liu & Rabinovich 2005), as well as FGF-2, a growth factor involved in tumor-induced angiogenesis (Bikfalvi et al. 1997), are secreted from a large variety of cells, and both constitutive secretion and regulated secretion have been reported (Hughes 1999, Nickel 2003, Prudovsky et al. 2003).

The members of the second group of unconventional secretory proteins normally localize in the cytoplasm or the nucleoplasm of cells, where they mediate well-characterized intracellular functions. However, in the presence of a specific external stimulus, they are released from cells to mediate functions distinct from their intracellular roles. Examples of cytoplasmic proteins that can become extracellular signaling molecules are thioredoxin, a cytoplasmic factor involved in redox balance (Arner & Holmgren 2000), and high-mobility group box 1 (HMGB-1), a

nuclear protein that binds to chromatin and regulates gene expression (Agresti & Bianchi 2003, Bianchi & Agresti 2005). Several cell types release both full-length and truncated forms of thioredoxin (Ericson et al. 1992, Rubartelli et al. 1992), and oxidative stress induces its secretion from T lymphocytes (Kondo et al. 2004). Although the molecular mechanism of export has remained elusive, extracellular functions of thioredoxin, including its role as a cytokine in inflammatory processes, have been well documented (Pekkari et al. 2000, 2001, 2003, 2005; Angelini et al. 2002; Nishinaka et al. 2002; Nakamura et al. 2006). Also, Schwertassek et al. (2007) identified a cell surface receptor whose redox state is catalytically controlled by extracellular thioredoxin. Extracellular thioredoxin also controls TRPC, a member of the TRP family of cation channels involved in a broad range of sensory processes such as pain perception and thermosensation (Flockerzi 2007, Venkatachalam & Montell 2007). Thioredoxin activates TRPC by breaking a disulfide bridge in an extracellular loop of the ion channel and, therefore, may be an endogenous chemical factor that is sensed by TRP channels (Xu et al. 2008).

Monocytes and macrophages, activated by inflammatory signals such as bacterial LPS and lysophosphatidylcholine, are the predominant sources of secreted HMGB-1 (Gardella et al. 2002). This observation is consistent with an extracellular role of HMGB-1 as a proinflammatory cytokine (Erlandsson et al. 1998; Wang et al. 1999, 2004b; Müller et al. 2001; Scaffidi et al. 2002; Degryse & de Virgilio 2003; Yang et al. 2005). Receptors, through which HMGB-1 signaling occurs, have been identified, and pharmacological manipulation of the corresponding signal transduction pathways improves survival in experimental sepsis (Wang et al. 2004a). A prerequisite for HMGB-1 secretion is its redistribution from the nucleus to the cytoplasm. HMGB-1 actively shuttles between the nucleus and the cytoplasm, with a steady-state localization in the nucleus. Upon LPS-induced activation of macrophages, HMGB-1 becomes hyperacylated, causing a shift of its steady-state localization to the cytoplasm. This process appears to be essential for the subsequent secretion of HMGB-1 (Bonaldi et al. 2003).

There are further examples of unconventional secretory proteins, such as annexins (Chapman et al. 2002, 2003; Gerke & Moss 2002; Danielsen et al. 2003; Peterson et al. 2003; Deora et al. 2004; Wein et al. 2004; Omer et al. 2006), the S100 family of $Ca^{2+}$ binding proteins (Davey et al. 2001, Landriscina et al. 2001b, Prudovsky et al. 2002, Mandinova et al. 2003, Flatmark et al. 2004), epimorphin (Radisky et al. 2003, Flaumenhaft et al. 2007, Hirai et al. 2007), Engrailed homeoproteins (Joliot et al. 1998; Maizel et al. 1999, 2002; Dupont et al. 2007), and an acyl-CoA binding protein required for terminal differentiation of spore cells in *Dictyostelium* development (Kinseth et al. 2007). The last protein represents an example of unconventional protein secretion in lower eukaryotes. In unicellular eukaryotes such as *S. cerevisiae*, it is not clear whether unconventional secretion of larger, soluble proteins exists. So far, the only known example of ER/Golgi-independent secretion in yeast is a-factor, a prenylated mating peptide whose secretion is mediated by the ABC transporter Ste6 (McGrath & Varshavsky 1989).

Additional examples of unconventional secretory proteins have recently been summarized elsewhere (Nickel 2003, Prudovsky et al. 2007). However, at this point, their extracellular functions and/or molecular details of their export mechanisms are poorly understood.

## Potential Intracellular Pathways of Unconventional Protein Secretion

Four principal mechanisms of intracellular trafficking have been proposed to mediate unconventional secretory pathways (Nickel 2005):

1. direct translocation from the cytoplasm across the plasma membrane into the extracellular space,
2. lysosomal secretion,

3. secretion by exosomes derived from multivesicular bodies (MVBs), and
4. secretion by plasma membrane blebbing and vesicle shedding.

As evident from this list, both vesicular and nonvesicular pathways of unconventional protein secretion have been proposed. Among the classical examples, FGF-1 and FGF-2 as well as interleukin 1α have been suggested to directly translocate across the plasma membrane (Tarantini et al. 2001, Prudovsky et al. 2002, Schäfer et al. 2004). By contrast, interleukin 1β was proposed to make use of secretory lysosomes (Andrei et al. 1999, 2004), a mechanism that has also been proposed for HMGB-1 (Gardella et al. 2002, Bonaldi et al. 2003). Galectin-1 and galectin-3 associate with the inner leaflet of plasma membranes and may be released in membrane-bound vesicles (Cooper & Barondes 1990, Mehul & Hughes 1997) by a process termed plasma membrane blebbing or shedding. Another secretory mechanism that releases vesicles into the medium is based on exosomes, which are internal vesicles of MVBs (a subset of endosomes). Although MVBs primarily deliver cytoplasmic proteins for degradation by fusion with lysosomes (Piper & Katzmann 2007), they also fuse with plasma membranes, releasing exosomes into the extracellular space (Murk et al. 2002, Stoorvogel et al. 2002, Fevrier & Raposo 2004).

**Possible mechanisms in interleukin 1β secretion.** Secretion of interleukin 1β may occur via multiple mechanisms. First, an inflammatory signal such as bacterial LPS activates monocytes, leading to the synthesis of the precursor form of interleukin 1β, which is not secreted. In a second step, another trigger such as extracellular ATP induces LPS-activated monocytes to convert the precursor form of interleukin 1β into the mature secretion-competent form. Caspase 1 mediates this processing event (Thornberry et al. 1992), and the overall signaling process that results in the secretion of interleukin 1β involves a multimeric protein complex termed the inflammasome (Burns et al. 2003, Ogura et al. 2006). Although these initial signaling events are not debated, the following steps, i.e., the molecular mechanism of secretion of the mature form of interleukin 1β, are controversial. All unconventional secretory mechanisms involving membrane-bound compartments (see above and Nickel 2005) have been suggested to play a role in interleukin 1β release. First, Rubartelli and coworkers reported evidence that the mature form of interleukin 1β is generated within lysosomes and that extracellular ATP, concomitant with an increase in cytoplasmic [$Ca^{2+}$], causes the fusion of secretory lysosomes with the plasma membrane (Andrei et al. 1999, 2004). This process results in the release of soluble interleukin 1β that directly affects its targets. An alternative view by Surprenant and coworkers suggests that ATP-dependent activation of P2X7 receptors mediates shedding of plasma membrane vesicles that are positive for extracellular interleukin 1β (MacKenzie et al. 2001). However, conflicting studies indicate that interleukin 1β secretion does not depend on ATP-induced plasma membrane blebbing concomitant with shedding of microvesicles (Brough et al. 2003, Verhoef et al. 2003). Another study also concludes that neither lysosomal secretion nor plasma membrane shedding represents a major pathway of interleukin 1β secretion (Qu et al. 2007). This latter conclusion was based on differential requirements for extracellular [$Ca^{2+}$] and differential sensitivities of interleukin 1β release and of plasma membrane shedding to pharmacological manipulations of primary macrophages. Moreover, Qu et al. (2007) provided evidence that, upon stimulation of P2X7 receptors, interleukin 1β is secreted as a luminal component of exosomes.

Thus, three molecular mechanisms have been suggested as the main pathways of interleukin 1β secretion: (*a*) lysosomal secretion, (*b*) plasma membrane shedding, and (*c*) exosomes derived from MVBs. It is presently unclear whether these data are indicative of several distinct and physiologically relevant pathways or whether the complexity of the experimental

systems and the functional overlap with other pathways, such as P2X7-induced cell death, prevent the identification of a single pathway with physiological relevance. An obvious problem is that only factors involved in the induction of interleukin 1β secretion have been identified. By contrast, molecular factors involved in translocation of interleukin 1β into endolysosomal compartments or sorting into membrane blebs remain elusive.

**Possible molecular mechanisms in FGF-2 secretion.** As for interleukin 1β (see previous subsection), investigators have suggested multiple export mechanisms for FGF-2 and galectin-1. Using an in vitro system employing plasma membrane inside-out vesicles, Schäfer et al. (2004) showed that FGF-2 and galectin-1 can directly translocate across plasma membranes. In line with these findings, heparan sulfate proteoglycans (HSPGs) directly facilitate FGF-2 secretion (Zehe et al. 2006). According to one proposed model, FGF-2 is initially recruited to the inner leaflet of plasma membranes, followed by membrane translocation in an HSPG-dependent manner (Nickel 2007). That cell surface receptors form a molecular trapping mechanism for unconventional secretory lectins has also been shown for galectin-1 (Seelenmeyer et al. 2005), and following secretion from various cell types, FGF-2 and galectin-1 are quantitatively retained on cell surfaces (Trudel et al. 2000, Seelenmeyer et al. 2005, Zehe et al. 2006). Thus, for both FGF-2 and galectin-1, researchers have described nonvesicular pathways of secretion that rely on direct protein translocation across plasma membranes (Nickel 2007).

Two additional modes of FGF-2 externalization have been proposed. Extensive plasma membrane blebbing, concomitant with shedding of vesicles into the extracellular space, may play a role in FGF-2 secretion (Taverna et al. 2003, Schiera et al. 2007, Proia et al. 2008). Vittorelli and colleagues reported that, upon serum starvation, SK-Hep1 cells release vesicles containing FGF-2 (Taverna et al. 2003). In these cells, however, FGF-2 expression does not induce plasma membrane blebbing. Rather, prolonged serum starvation followed by a serum shock was used to induce plasma membrane blebbing. Quantitative analysis of the relative amounts of FGF-2 released in shed vesicles and controls for a potential concomitant release of unrelated cytoplasmic proteins have not been performed (Taverna et al. 2003). Another molecular mechanism potentially involved in FGF-2 secretion relates to the finding that the expression of the Epstein-Barr virus protein LMP1 results in increased FGF-2 export efficiency (Wakisaka et al. 2002). Recently, Ceccarelli et al. (2007) proposed that LMP-1-dependent release of FGF-2 involves exosomes derived from MVBs. Thus, besides direct membrane translocation, investigators have suggested two additional modes to play a role in FGF-2 secretion that are based on FGF-2 export in cell-derived membrane vesicles.

These controversies have been addressed in a recent study using a cellular model system in which efficient export of both FGF-2 and galectin-1 was demonstrated (Cho & Cummings 1995, Engling et al. 2002, Backhaus et al. 2004, Seelenmeyer et al. 2005, Zehe et al. 2006). Although exposing significant amounts of FGF-2 and galectin-1 on their surfaces, these cells did not form plasma membrane blebs (Seelenmeyer et al. 2008). These cells do not release vesicles into the cell culture supernatant carrying FGF-2 or galectin-1, nor do they release soluble forms of these proteins. Finally, this study (Seelenmeyer et al. 2008) showed that the Rho kinase inhibitor Y27632, which blocks plasma membrane blebbing, has no impact on FGF-2 secretion efficiency. Similar results were obtained for galectin-1. In the light of these data, it seems likely that the findings of Vittorelli and coworkers (Taverna et al. 2003) are related to the induction of apoptosis, a process that is induced by serum deprivation (Fuhrmann et al. 2001, Schamberger et al. 2005). Although Taverna et al. (2003) claim that FGF-2 vesicles being shed into the cell culture supernatant were not derived from apoptotic cells, apoptotic programs may have been induced under the

experimental conditions described. Perhaps the experimental conditions being used in this study did not result in cell death because FGF-2 inhibited certain stages in the progression of apoptotic programs (Schamberger et al. 2004). Finally, the fact that Taverna et al. (2003) were able to use annexin V–coupled beads to purify these vesicles, i.e., these vesicles present phosphatidylserine on their surfaces, also supports the conclusion that they are derived from cells in which apoptosis was induced. On the basis of these considerations, plasma membrane shedding of FGF-2-containing vesicles may not play a role in FGF-2 secretion under physiologically relevant conditions.

With regard to a potential role of exosomes in FGF-2 secretion (Ceccarelli et al. 2007), it is again not clear whether these findings are of physiological relevance. One issue is that the authors make use of model systems in which FGF-2 secretion cannot be observed at all when LMP-1 is absent. Additionally, the amounts of FGF-2 being found in exosomes were not quantified, and therefore it is difficult to compare them with the amounts of FGF-2 that associate with cell surfaces in other well-established model systems (Trudel et al. 2000, Engling et al. 2002, Backhaus et al. 2004, Zehe et al. 2006). Another issue is that, owing to the fact that flotation analyses have not been performed (Ceccarelli et al. 2007), it is not clear whether FGF-2 found in sediments derived from cell culture supernatants is associated solely with membrane vesicles or also reflects material present in protein aggregates.

In the case of galectin-1, early studies proposed plasma membrane blebbing as a potential mechanism of secretion occuring during the differentiation of myoblasts into myotubes (Cooper & Barondes 1990). As opposed to other model systems in which secreted galectin-1 is retained on cell surfaces (Seelenmeyer et al. 2003, 2005), myotubes release galectin-1 into the medium (Cooper & Barondes 1990). Even though CHO cells efficiently secrete galectin-1, resulting in their association with cell surfaces (Cho & Cummings 1995; Seelenmeyer et al. 2003, 2005), galectin-1 does not induce plasma membrane blebbing in these cells (Seelenmeyer et al. 2008). These discrepancies may be due to differences in cell processing for imaging purposes. In the studies of Cooper & Barondes (1990), cells were fixed prior to analysis, a treatment that causes both membrane blebbing and the release of vesicles into the extracellular space (Scott 1976). Therefore, in the more recent study (Seelenmeyer et al. 2008), galectin-1-expressing cells were analyzed by live-cell imaging, and plasma membrane blebbing was undetectable. Thus, it is not clear whether plasma membrane blebbing observed in fixed cells is related to physiologically relevant processes.

From the combined data discussed above, we conclude that efficient secretion of FGF-2 and galectin-1 in a well-characterized model system such as CHO cells is not mediated by either plasma membrane blebbing or the release of exosomes derived from MVBs. Rather, direct translocation across the plasma membrane, requiring cell surface counter receptors, appears to mediate unconventional secretion of these factors (Seelenmeyer et al. 2005, 2008; Zehe et al. 2006; Nickel 2007).

## Molecular Mechanism of FGF-2 Membrane Translocation as a Paradigm for Unconventional Protein Secretion

Among secretory proteins exported by unconventional means, FGF-2 is currently the best-characterized example with regard to the molecular mechanisms involved. As outlined below, its subcellular site of membrane translocation, *cis*-elements, and interacting molecules required for FGF-2 sorting into its unusual secretory pathway as well as folding aspects and energy requirements have been addressed recently.

**Known key features of FGF-2 secretion.** Investigators have elucidated a number of key features of the overall process of FGF-2 secretion from cells. As revealed by an in vitro system, FGF-2 membrane translocation requires

neither ATP hydrolysis nor a membrane potential (Schäfer et al. 2004). Cytoplasmic FGF-2 is not incorporated into transport vesicles but rather directly traverses the plasma membrane to access the extracellular space (Schäfer et al. 2004, Zehe et al. 2006). HSPGs provide the membrane-proximal binding sites required for FGF-2 export (Zehe et al. 2006, Nickel 2007). These data suggest that FGF-2 translocation across the plasma membrane is a diffusion-controlled process mediated by either a proteinaceous transporter or a so-far-unrecognized ability of FGF-2 to pass through the plasma membrane by a transporter-independent mechanism. These findings have led to the conclusion that HSPGs form an extracellular molecular trap that is essential for both directional transport and storage of FGF-2 in the extracellular space (Zehe et al. 2006, Nickel 2007). This aspect of the FGF-2 export mechanism appears to be similar to another unconventional secretory protein, galectin-1 (Seelenmeyer et al. 2005).

**FGF-2 targeting to the inner leaflet of plasma membranes as the entry point of the FGF-2 secretory pathway.** A major aspect of recent studies was to elucidate the molecular mechanism by which FGF-2 export is initiated, i.e., the factors that mediate a transient interaction with the inner leaflet of plasma membranes. In addition to having multiple functions in cellular physiology (Di Paolo & De Camilli 2006), PIPs have been identified as landmarks for cytoplasmic proteins that associate with a specific subcellular membrane (Behnia & Munro 2005). The PIP $PI(4,5)P_2$ has been specifically implicated in the recruitment of cytosolic proteins to the inner leaflet of the plasma membrane (McLaughlin et al. 2002). Various recognition motifs for PIPs, including polybasic clusters (Heo et al. 2006), have been described (Ellson et al. 2002, Stenmark et al. 2002, Lemmon 2003).

That FGF-2 contains a relatively large number of lysine and arginine residues near its C terminus led to the hypothesis that FGF-2 might be able to bind PIPs. Further evidence for this hypothesis came from the observation that a phosphate ion cocrystallized with recombinant FGF-2 coordinated by amino acid residues Asn 35, Arg 128, and Lys 133 (Kastrup et al. 1997). Starting from these observations, a recent study showed that FGF-2 efficiently binds to liposomes containing $PI(4,5)P_2$ (Temmerman et al. 2008). $PI(4,5)P_2$ is found mainly in plasma membranes, whereas other PIPs are enriched in the Golgi complex [PI(4)P], early endosomes [PI(3)P], or late endosomes [$PI(3,5)P_2$] (Di Paolo & De Camilli 2006). Therefore, the observed interaction of FGF-2 with $PI(4,5)P_2$ is also consistent with studies demonstrating that the plasma membrane is the subcellular site of FGF-2 membrane translocation (Schäfer et al. 2004, Zehe et al. 2006). Intriguingly, the interaction of FGF-2 with $PI(4,5)P_2$ depends on a lipid background resembling plasma membranes; only background levels of FGF-2 binding were observed when $PI(4,5)P_2$ was reconstituted in liposomes made of phosphatidylcholine. Omission of cholesterol and sphingomyelin also reduced binding of FGF-2 to $PI(4,5)P_2$-containing liposomes. These and other data, as well as the fact that $PI(4,5)P_2$ is strongly enriched in cholesterol-dependent lipid microdomains (McLaughlin et al. 2002), led to the proposal that $PI(4,5)P_2$ clustering within a specialized membrane domain may be required for FGF-2 recruitment to plasma membranes. This view is consistent with a failure of $PI(4,5)P_2$ to form clusters in a pure phosphatidylcholine lipid background at physiological concentrations (Fernandes et al. 2006). Two findings anchor the conclusion that the interaction of $PI(4,5)P_2$ with FGF-2 is of biological relevance. First, RNAi-mediated downregulation of type I PIP kinases reduces $PI(4,5)P_2$ levels by 90% and significantly impairs FGF-2 secretion. Second, FGF-2 variant forms that fail to bind $PI(4,5)P_2$ are also secreted inefficiently. Taken together, these data demonstrate that binding to $PI(4,5)P_2$ at the inner leaflet of plasma membranes represents the entry point of the unconventional secretory pathway of FGF-2 (Temmerman et al. 2008).

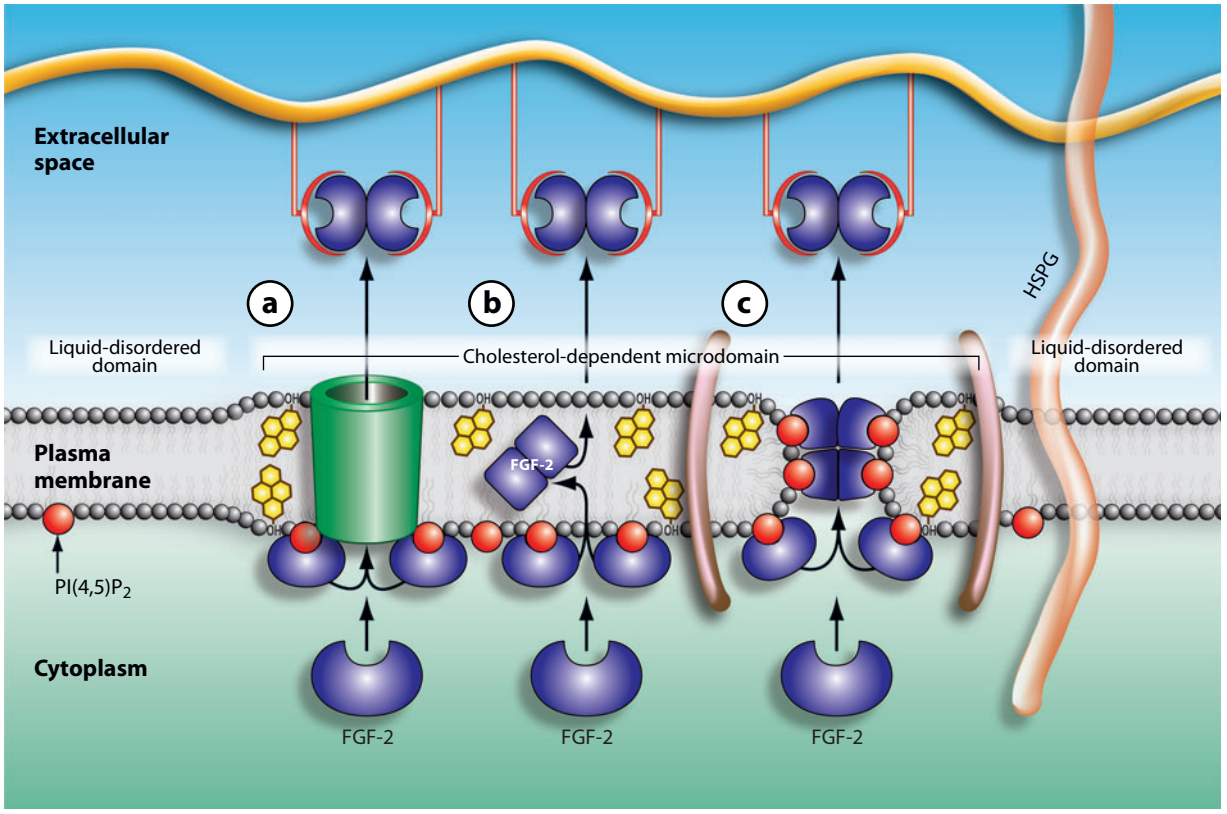



**Figure 2**

Potential mechanisms of fibroblast growth factor-2 (FGF-2) membrane translocation. (*a*) FGF-2 membrane translocation mediated by a protein-conducting channel. In this model, FGF-2 is recruited to the inner leaflet by binding to phosphatidylinositol 4,5 bisphosphate [$PI(4,5)P_2$]. Transport within the channel is diffusion controlled, and HSPG-mediated trapping ensures net transport into the extracellular space. (*b*) FGF-2 membrane translocation based on a $PI(4,5)P_2$-induced conformational change that allows for penetration of the lipid bilayer. (*c*) FGF-2 membrane translocation based on the generation of a hydrophilic pore whose opening is initiated by multivalent interactions of FGF-2 with $PI(4,5)P_2$ enriched within a cholesterol-dependent lipid microdomain. Both positive membrane curvature induced by high concentrations of $PI(4,5)P_2$ and an additional proteinaceous factor may facilitate this process. HSPG denotes heparan sulfate proteoglycan.

**Working hypotheses on how FGF-2 physically traverses plasma membranes as a key aspect of its unconventional secretory route.** **Figure 2** illustrates various molecular aspects of the unconventional secretory pathway of FGF-2, such as $PI(4,5)P_2$-dependent recruitment of FGF-2 to the inner leaflet of plasma membranes and an extracellular trapping mechanism mediated by HSPGs. A key aim of future research will be to elucidate the molecular mechanism by which FGF-2 physically traverses the plasma membrane. One obvious option would be a plasma membrane–resident transporter forming a protein-conducting channel with specificity for FGF-2 (**Figure 2*a***). Such a pore might allow FGF-2 membrane translocation by passive diffusion and could work in conjunction with both $PI(4,5)P_2$ at the inner leaflet and the HSPG-mediated molecular trap at the extracellular side. So far, such a transporter has not been identified; however, genome-wide screening procedures based on RNAi-mediated gene silencing might reveal such a gene product.

Alternatively, an as-yet-unrecognized property of FGF-2 may enable its passage through

the membrane by a transporter-independent process. There are two possible mechanisms, both of which are likely to depend on the interaction of FGF-2 with $PI(4,5)P_2$ at the inner leaflet of the plasma membrane (**Figure 2*b*,*c***). One possibility would be that this interaction causes a conformational change in FGF-2 that allows for its transient solubilization within the hydrophobic core of the membrane (**Figure 2*b***). Interactions of proteins with $PI(4,5)P_2$ at a membrane interface can result in conformational changes (Milburn et al. 2003). In this scenario, FGF-2 may exit the plasma membrane on the extracellular side by binding to HSPGs, a process that would be accompanied by another conformational change back to its water-soluble structure. With regard to solubilization within a hydrophobic environment, molten globule conformations may play a role (Rajalingam et al. 2007). Additionally, in certain proteins, such conformations may be induced in an acidic environment such as the interface between the cytoplasm and a plasma membrane lipid microdomain in which PIPs like $PI(4,5)P_2$ are strongly enriched (Prudovsky et al. 2007). However, in the case of FGF-2, this hypothesis is very difficult to reconcile with some of the experimental data on FGF-2 secretion. In various model systems, FGF-2 can be fused to a variety of other protein domains such as green fluorescent protein (GFP) and dihydrofolate reductase (DHFR) without an appreciable loss of export efficiency (Florkiewicz et al. 1995, Engling et al. 2002, Backhaus et al. 2004). In particular, the DHFR domain is characterized by a very stable fold in the presence of its ligands methotrexate or aminopterin, and therefore import of corresponding fusion proteins into mitochondria is inhibited under such experimental conditions (Eilers & Schatz 1986, Wienhues et al. 1991). By contrast, the addition of the DHFR domain to FGF-2 was compatible with FGF-2 secretion in the presence of aminopterin (Backhaus et al. 2004). These findings make it rather unlikely that FGF-2 secretion involves an intermediate that is transiently dissolved in the hydrophobic core of the membrane because it is difficult to reconcile how the GFP or the aminopterin-stabilized DHFR domain could be accommodated in this process.

**Figure 2*c*** illustrates a second potential mechanism for FGF-2 membrane translocation in a transporter-independent manner. This hypothesis is again centered around the interaction of FGF-2 with $PI(4,5)P_2$ as the entry point of this secretory pathway. On the basis of the binding studies discussed above, Temmerman et al. (2008) concluded that lipid microdomains involved in FGF-2 recruitment may contain as much as 30 mol% of $PI(4,5)P_2$. Such microdomains containing unusually high amounts of $PI(4,5)P_2$ may be preexisting, or FGF-2 binding may cause an additional enrichment of $PI(4,5)P_2$ in these membrane structures. In either case, the result would be a unique membrane structure. In particular, owing to its bulky head group relative to its slim hydrophobic part, $PI(4,5)P_2$ can be classified as a so-called nonbilayer lipid (McMahon & Gallop 2005). This is because, in a regular lipid bilayer made from phosphatidylcholine, $PI(4,5)P_2$ present in just one monolayer cannot be accommodated in the absence of membrane curvature. In the context of the plasma membrane, in which $PI(4,5)P_2$ localizes exclusively to the inner leaflet, domains containing large amounts of $PI(4,5)P_2$ are characterized by positive membrane curvature, i.e., curvature directed toward the cytoplasm (McMahon & Gallop 2005).

At this point our knowledge about the ultrastructure of cholesterol-dependent and $PI(4,5)P_2$-enriched lipid microdomains is quite limited. We can only speculate on the structural consequences of FGF-2 binding to such domains. An interesting point to keep in mind is that extracellular FGF-2 has the intrinsic ability to oligomerize (Facchiano et al. 2003). Intracellular FGF-2 may oligomerize as well, maybe even as a result of its interaction with $PI(4,5)P_2$ at the inner leaflet of plasma membranes. Thus, this process would render FGF-2 multivalent with regard to $PI(4,5)P_2$ binding. An exciting hypothesis is that, on the basis of positive membrane curvature due to high concentrations of $PI(4,5)P_2$ coordinated through multivalent interactions between $PI(4,5)P_2$ and FGF-2

molecules, a transient and highly ordered structure may be established; such a structure can provide a hydrophilic environment through which FGF-2 can translocate across the plasma membrane (**Figure 2*c***). Accessory proteins yet to be identified may help to stabilize such a membrane structure, which is likely to occur only transiently. In this scenario, HSPGs trap FGF-2 molecules on the extracellular side, ensuring directional transport toward the extracellular space. Because it is likely that FGF-2 binding to PI(4,5)$P_2$ and HSPGs is mutually exclusive, HSPGs may play an even more active role by extracting FGF-2 molecules bound to PI(4,5)$P_2$ at the membrane. This mechanism of transport would explain the observations that FGF-2 secretion does not depend on ATP hydrolysis (Schäfer et al. 2004) and that FGF-2 membrane translocation is compatible with tags such as GFP and DHFR domains (Engling et al. 2002, Backhaus et al. 2004, Zehe et al. 2006). This hypothesis remains highly speculative and needs to be challenged by new approaches such as in vitro reconstitution of FGF-2 membrane translocation with chemically defined components as well as by biophysical methods such as solid-state nuclear magnetic resonance to elucidate ultrastructural aspects of the interaction of FGF-2 with PI(4,5)$P_2$-enriched lipid microdomains.

## CONCLUSIONS

In addition to transport along the classical secretory pathway, eukaryotic cells employ various alternative routes for cell surface delivery of both soluble and membrane proteins. Although all these pathways share the commonality of not requiring a functional Golgi apparatus, they otherwise appear to be different in many regards. On the one hand, direct transfer of membrane proteins from peripheral ER domains to the plasma membrane occurs in a Golgi-independent manner. On the other hand, researchers have described more than 20 soluble proteins that can be secreted from cells by mechanisms that involve neither the ER nor the Golgi apparatus. The molecular mechanisms of these unusual pathways are now beginning to emerge, and these long-standing problems in molecular cell biology may be solved in the years to come.

## DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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