The non-classical export routes: FGF1 and IL-1α point the way

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Summary

Non-classical protein release independent of the ER-Golgi pathway has been reported for an increasing number of proteins lacking an N-terminal signal sequence. The export of FGF1 and IL-1α, two pro-angiogenic polypeptides, provides two such examples. In both cases, export is based on the Cu^{2+}-dependent formation of multiprotein complexes containing the S100A13 protein and might involve translocation of the protein across the membrane as a 'molten globule'. FGF1 and IL-1α are involved in pathological processes such as restenosis and tumor formation. Inhibition of their export by Cu^{2+} chelators is thus an effective strategy for treatment of several diseases.

Key words: Fibroblast growth factor 1, FGF1, Interleukin 1α, IL-1α, Release, Non-classical, Copper, Synaptotagmin 1, S100A13

Introduction

Many biological processes involve polypeptide translocation across phospholipid membranes. Among them are export to the extracellular milieu (Blobel, 1995), transport in and out of the nucleus (Weis, 2003), and import into mitochondria (Endo et al., 2003; Gordon et al., 2000) and peroxisomes (Holroyd and Erdmann, 2001). These processes use specific transporters and frequently involve transmembrane pores and channels. The proteins translocated usually possess appropriate signal sequences and, in the case of classical protein secretion, this is a hydrophobic N-terminal sequence that allows the protein to enter the ER-Golgi pathway (Blobel, 2000).

However, several extracellular proteins lack signal sequences, and their export proceeds through endoplasmic reticulum (ER)-Golgi-independent non-classical routes (Table 1) (reviewed by Nickel, 2003). Currently, we know little about the export mechanisms of most of these proteins, the only two common features being the absence of a signal sequence in the protein and the insensitivity of the pathway to brefeldin A [a drug that specifically inhibits ER-to-Golgi transport (Misumi et al., 1986)]. Nevertheless, what is apparent is that non-classical export is not a single pathway but instead comprises several transport mechanisms. Here, we concentrate on two biologically important and functionally related proteins, fibroblast growth factor 1 (FGF1) and interleukin (IL)-1α, and their non-classical export pathways.

Structure and function of FGF1 and IL-1α

FGF1 and FGF2 are prototypical members of the FGF family (Szebenyi and Fallon, 1999). FGFs have a wide variety of biological activities. During embryogenesis, these growth factors regulate mesodermal induction, neurulation, and the formation of the circulatory and skeletal systems (Friesel and Maciag, 1999). Subsequently, they play a crucial role in angiogenesis, tissue regeneration, inflammation and the formation of some tumors (Friesel and Maciag, 1999). Their biological effects are mediated through activation of four transmembrane phosphotyrosine kinase receptors (FGFR1-4), with the participation of cell-surface heparan sulfate proteoglycans (HSPGs), and consequently require release of the polypeptide (Friesel and Maciag, 1999). Most members of the family therefore possess classical signal sequences but FGF1 and FGF2 are devoid of such sequences and thus are released by novel secretion mechanisms (Coulier et al., 1997; Friesel and Maciag, 1999).

The existence of FGF1- and FGF2-specific secretion pathways might represent a protective mechanism developed in the course of evolution and might be related to their high mitogenic potential and widespread expression. Indeed, a recombinant derivative of FGF1 that has an attached N-terminal signal sequence is a potent oncoprotein (Forough et al., 1993). Significantly, the FGFs of *Caenorhabditis elegans* and *Drosophila* have signal sequences (Coulier et al., 1997). Apparently, strictly programmed mosaic development of these organisms can rely on the regulation of FGF availability solely at the level of its expression. The more complicated and less hierarchical developmental strategies of chordates probably required the evolution of signal-peptide-less FGFs, whose accessibility might be more flexibly regulated post-translationally.

The IL-1 family (Dinarello, 1996; Stylianou and Saklatvala, 1998), of which IL-1α and IL-1β are prototypical members, numbers at least ten proteins. Nine of these, including IL-1α and IL-1β, do not have signal sequences despite acting through transmembrane receptors and thus requiring export (Dinarello, 1998; Stylianou and Saklatvala, 1998). These proteins are potent pro-inflammatory cytokines (Dinarello, 1996), inducing

biosynthesis of a variety of inflammation-related molecules, such as tumor necrosis factor (TNF), transforming growth factor (TGF)-β, granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), cyclooxygenase 2, endothelin-1, phospholipase A2, and inducible nitric oxide synthase (Dinarello, 1996). Unlike the FGFs, IL-1s are synthesized as higher molecular weight precursor (p) proteins. pIL-1α is cleaved by calpain or calpain-like proteases to form mature (m) IL-1α, and pIL-1β is cleaved by the IL-1-converting enzyme to form mIL-1β (Dinarello, 1996; Stylianou and Saklatvala, 1998).

Comparison of the crystal structures of FGF1, FGF2, IL-1α and IL-1β (Graves et al., 1990; Venkataraman et al., 1999; Zhu et al., 1991) reveals that they have very similar folds in spite of their very low sequence similarity (Fig. 1). These proteins contain β-barrel structures that are often found in transmembrane proteins, including bacterial pore-forming proteins (Chen and Funk, 2001; Heuck et al., 2000; Montoya and Gouaux, 2003) and are crucial for membrane insertion of some proteins (Heuck et al., 2000). This provided the first indication that similarities might exist between the release mechanisms of the IL-1 and FGF prototypes. It was especially interesting to compare the release of IL-1α and FGF1 since these two proteins have antagonistic effects upon the proliferation and migration of endothelial cells (Maier et al., 1990; Friesel and Maciag, 1999).

Cell stress induces FGF1 and IL-1α release

Under normal conditions, cells expressing FGF1 and IL-1α do not release these proteins. However, several stresses, such as heat shock (Jackson et al., 1992), hypoxia (Mouta Carreira et al., 2001), cultivation under low serum conditions (Shin et al., 1996) and cell treatment with low-density lipoproteins (LDLs) (Ananyeva et al., 1997), induce release of FGF1 from NIH 3T3 cells. Heat shock induces the export of mIL-1α from human promonocytic leukemia cells and activated peripheral mononuclear cells (Tarantini et al., 2001; Mandinova et al., 2003). The two latter cell types also exhibit heat-shock-induced export of pIL-1α (Mandinova et al., 2003). Interestingly, similarly to FGF2 (Shi et al., 1997), pIL-1α is not secreted from stressed NIH 3T3 cells (Tarantini et al., 2001). The retention of pIL-1α is most likely because of the nuclear localization sequence (Wessendorf et al., 1993) in its cleavable N-terminal precursor domain. Although pIL-1α is cleaved in monocytes/macrophages (Dinarello, 1992;

Table 1. Proteins exported through non-classical pathways

Protein	Reference	Signal peptide	Export sensitivity to brefeldin A	Release characteristics
Secretory transglutaminase	Aumuller et al., 1999	–	Insensitive	Constitutive, through membrane blebbing
Thioredoxin	Rubartelli et al.,1992; Rubartelli et al., 1995; Angelini et al., 2002	–	Insensitive	Induced by antigen-specific T cells, intracellular vesicles not involved
Galectins	Hughes, 1999; Sato et al., 1993; Lindstedt et al., 1993	–	Insensitive	Constitutive, through membrane blebbing
Il-1α	Tarantini et al., 2001; Mandinova et al., 2003	–	Insensitive	Stress-induced, Cu^{2+}-dependent, in complex with S100A13
Il-1β	Rubartelli et al., 1990; Andrei et al., 1999	–	Insensitive	Stress-induced, ABC-transporter-dependent, through the endolysosomal pathway
FGF1	Jackson et al., 1992; Tarantini et al., 1998; LaVallee et al., 1998; Mouta Carreira et al., 1998; Landriscina et al., 2001a; Landriscina et al., 2001b	–	Insensitive	Stress-induced, Cu^{2+}-dependent, in complex with S100A13 and p40 Syt1
FGF2	Florkiewicz et al., 1995; Mignatti et al., 1992; Engling et al., 2002	–	Insensitive	Constitutive, Na^+/K^+ ATPase-dependent
Sphingosine kinase	Ancellin et al., 2002	–	Insensitive	Constitutive, inhibited by cytochalasin
Annexin I	Chapman et al., 2003	–	Not tested	Glucocorticoid-induced, ABC-transporter-dependent
Annexin II	Peterson et al., 2003	–	Not tested	Thrombin-induced, in complex with p11
p40 Synaptotagmin 1	LaVallee et al., 1998; Tarantini et al., 1998	–	Insensitive	Constitutive
S100A13	Landriscina et al., 2001a; Landriscina et al., 2001b	–	Insensitive	Constitutive
HIV Tat	Chang et al., 1997	–	Insensitive	Constitutive
Herpes VP 22 protein	Elliott and O'Hare, 1997	–	Insensitive	Constitutive
Foamy virus Bet protein	Lecellier et al., 2002	–	Insensitive	Constitutive
Engrailed 2	Joliot et al., 1998; Maizel et al., 1999; Maizel et al., 2002	–	Insensitive	Attenuated by the CK2-dependent phosphorylation
HMGB1	Gardella et al., 2002; Passalacqua et al., 1997; Passalacqua et al., 1998; Sparatore et al., 1996	–	Insensitive	Stress-induced, through an endolysosomal pathway
Leishmania HASPB protein	Denny et al., 2000	–	Insensitive	Constitutive, acylation-dependent

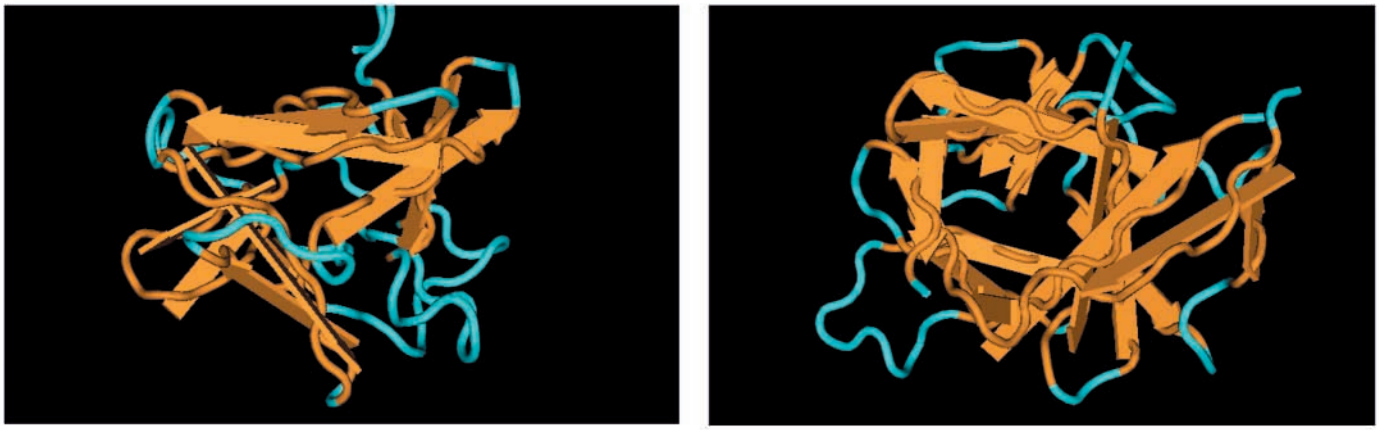

Fig. 1. Three-dimensional representation of the β-barrel structures of human mIL-1α (Graves et al., 1990) and human FGF1 (Lozano et al., 2000). β-sheet domains are indicated in yellow and are depicted as rotating counter clockwise around the open centers of the structures. The structures were downloaded from the Protein Data Bank of the NCBI (http://www.rcsb.org/pdb/).

Dinarello, 1996), the cleavage does not occur in NIH 3T3 cells (Tarantini et al., 2001), perhaps because of high levels of the calpain inhibitor calpastatin (Goll et al., 2003) in the cytosol of these cells. In macrophage-like cells, the control of pIL-1α nuclear localization is less stringent since these cells display pIL-1α both in the nucleus and in the cytoplasm (Beuscher et al., 1988; Kobayashi et al., 1990).

Significantly, co-expression of pIL-1α and FGF1 in NIH 3T3 cells inhibits the stress-induced release of FGF1 (Tarantini et al., 2001). It appears that the release pathways used by FGF1 and IL-1α interact. Thus, pIL-1α could bind some important protein(s) shared by these pathways and sequester it in the nucleus. Indeed, FGF1 and IL-1α release pathways share several similarities, including the delayed character of export, which becomes detectable only after 90 minutes of stress (Jackson et al., 1992; Tarantini et al., 2001). This delay presumably reflects the need for stress-induced synthesis of proteins that participate in the export of IL-1α and FGF1 because both IL-1α release and FGF1 release are sensitive to inhibition of transcription and translation (Jackson et al., 1992; Tarantini et al., 2001).

FGF2 and IL-1β also exhibit non-classical release. However, their export mechanisms appear to be quite different. Unlike FGF1 and IL-1α, FGF2 is exported constitutively (Florkiewicz et al., 1995; Mignatti et al., 1992). The release of FGF2 is highly sensitive to the inhibitors of Na^+/K^+ ATPase (Dahl et al., 2000; Florkiewicz et al., 1998), whereas the export of FGF1 is refractory to these compounds (F.T., I.P. and T.M., unpublished).

The release of IL-1β is induced by lipopolysaccharides (Andrei et al., 1999; Rubartelli et al., 1990) but not by heat shock (A.M. and T.M., unpublished). In addition, unlike the export of FGF1 and IL-1α (Jackson et al., 1992) (F.T., I.P. and T.M., unpublished), IL-1β release is sensitive to methylamine (Rubartelli et al., 1990), an inhibitor of exocytosis, and to sulfonylurea glybenclamide, an inhibitor of the mammalian ATP-binding cassette (ABC) translocator ABC1 (Andrei et al., 1999). Another notable difference is that, unlike FGF1 and IL-1α, which are distributed homogeneously in the cytoplasm (Prudovsky et al., 2002), IL-1β is contained within cytoplasmic vesicles expressing lysosomal but not ER-Golgi markers (Andrei et al., 1999). Export of IL-1β thus appears to be based on its intracellular translocation into lysosome-like vesicles and the subsequent exocytotic fusion of these vesicles with the cell membrane (Andrei et al., 1999). It will be interesting to determine whether the constitutive release of FGF2 is also sensitive to these pharmacological agents. Thus, in spite of the very similar 3D structures of FGF1 and IL-1 prototypes, FGF2 and IL-1β appear to be secreted through pathways different from IL-1α and FGF1.

The stress-mediated intracellular transport of FGF1 and IL-1α

The inhibition of FGF1 and IL-1α release by 2-deoxyglucose (Jackson et al., 1992; Tarantini et al., 2001) demonstrates that these pathways are dependent on ATP. In addition, an intact actin cytoskeleton is important, since release of FGF1 and IL-1α is sensitive to agents that attenuate actin stress fibers, such as latrunculin and amlexanox (Landriscina et al., 2000; Mouta Carreira et al., 1998; Tarantini et al., 2001). By contrast, microtubule inhibitors, such as nocodazole, fail to inhibit FGF1 release (F.T., I.P. and T.M., unpublished). Real-time confocal studies of cells transfected with an FGF1-GFP chimera have demonstrated stress-induced migration of cytosolic FGF1 to the cell membrane 60 minutes after heat shock and this translocation can be completely inhibited by amlexanox (Prudovsky et al., 2002). Likewise, heat shock also induces translocation of an IL-1α-RFP chimera from the cytosol to the cell membrane (Mandinova et al., 2003). Although the actin cytoskeleton transports different types of cytoplasmic membrane vesicle (Rogers and Gelfand, 2000), at least at the level of fluorescence microscopy, neither FGF1 nor IL-1α appears to be present in vesicular structures under normal conditions or during heat shock (Prudovsky et al., 2002).

The stress-induced formation of multiprotein FGF1 and IL-1α release complexes

FGF1 is released during stress as a covalent cysteine-linked

homodimer (Jackson et al., 1992). The evolutionarily conserved Cys30 residue is crucial for its stress-mediated release (Tarantini et al., 1995). Interestingly, the FGF1 homodimer exhibits a low heparin affinity (compared with the monomer), as well as low mitogenic activity in vitro (Engleka and Maciag, 1992). Dimer formation might therefore be a way of storing and possibly transporting FGF1 in an inactive form. However, low heparin affinity makes dimeric FGF1 potentially more susceptible to proteolysis, since heparin and HSPGs protect FGF family members from proteases (Friesel and Maciag, 1999; Rosengart et al., 1988). The balance between the monomeric and dimeric forms of FGF1 in the extracellular compartment might be regulated by stress-induced extracellular reducing and oxidizing agents. For example, the reducing agent thioredoxin is a signal-peptide-less protein released through a non-classical pathway (Rubartelli et al., 1992; Rubartelli et al., 1995), and Cu^{2+} ions are potential extracellular oxidizing agents that could convert reduced monomeric FGF1 to the dimeric form. Indeed, in a cell-free system, Cu^{2+} efficiently induces FGF1 dimerization (Engleka and Maciag, 1992). However, the role of intracellular Cu^{2+} in the release of FGF1 and IL-1α is even more significant (see below).

IL-1α release does not appear to depend on covalent dimerization. mIL-1α is exported as a monomeric, biologically active cytokine (Mandinova et al., 2003; Tarantini et al., 2001). Moreover, it has no evolutionarily conserved equivalent of Cys30 in FGF1 (Furutani et al., 1986; Lomedico et al., 1984), and a cysteine-free IL-1α mutant is released normally in response to cellular stress (A.M., I.P. and T.M., unpublished).

Both mIL-1α and FGF1 are exported as components of multiprotein release complexes that, at least in the case of FGF1, assemble near the inner surface of the plasma membrane (Prudovsky et al., 2002). The first evidence for such complexes resulted from HPLC analysis of high-molecular-weight FGF1-containing fractions from bovine and ovine brains (Maciag et al., 1982; Mouta Carreira et al., 1998). Brain-derived FGF1 is associated with at least four other polypeptides, which include S100A13 and the p40 form of synaptotagmin 1 (Syt1) (Burgess et al., 1985; Mouta Carreira et al., 1998). S100A13 belongs to the S100 family of polypeptides, which are small acidic proteins that have two Ca^{2+}-binding EF-hand domains (Heizmann et al., 2002). The biological functions of most S100s are not defined but, significantly, the intracellular distributions and/or expression levels of some family members are modified in response to cellular stress (Breen et al., 1999; Du et al., 2002; Duarte et al., 1999; Hoyaux et al., 2000; Hsieh et al., 2002; Kucharczak et al., 2001; Lam et al., 2001; Mandinova et al., 1998; Migheli et al., 1999; Zhang et al., 2002). A specific structural characteristic of S100A13 is the presence of a C-terminal domain rich in basic residues (Wicki et al., 1996). The expression of S100A13 in NIH 3T3 cells is detectable by RT-PCR analysis (Landriscina et al., 2001a).

All S100 proteins lack classical signal sequences, but at least some of them are released into the extracellular compartment (Heizmann and Cox, 1998). S100A13 transfected into NIH 3T3 cells is constitutively released (Landriscina et al., 2001a); however, when it is co-expressed with either FGF1 or mIL-1α, its release becomes stress-dependent (Landriscina et al., 2001a; Mandinova et al., 2003). This observation in conjunction with experiments using a dominant-negative S100A13 deletion mutant lacking the basic C-terminal domain demonstrated that S100A13 is an indispensable part of the multiprotein FGF1 release complex (Landriscina et al., 2001a). Similar experiments provided evidence that S100A13 expression is also critical for IL-1α release (Mandinova et al., 2003). Interestingly, although a cysteine-free FGF1 mutant is not released in response to stress (Tarantini et al., 1995), its co-expression with S100A13 results in the stress-induced export of both proteins (Landriscina et al., 2001a). It appears that overexpression of S100A13 induces the non-covalent dimerization of cysteine-free FGF1.

The p40 Syt1 component of the brain-derived FGF1-containing multiprotein complex represents the extravesicular portion of the transmembrane p65 Syt1 protein. Syt1 participates in the docking of a variety of secretory vesicles, including synaptic vesicles, at the cell membrane prior to their subsequent exocytosis (Sudhoff and Rizo, 1996). Similarly to other members of the synaptotagmin protein family, Syt1 displays two Ca^{2+}-binding C2 domains in its extravesicular portion (Marqueze et al., 2000). p40 Syt1 is believed to be produced by proteolytic cleavage of p65 near its transmembrane domain (Marqueze et al., 2000; Sudhoff and Rizo, 1996). In contrast to p65 Syt1, which displays a classical N-terminal signal peptide in its primary structure and localizes primarily to the ER-Golgi apparatus, cytoplasmic vesicles and cell membrane, signal-peptide-less p40 Syt1 displays a diffuse cytosolic distribution (C.B., I.P. and T.M., unpublished). Interestingly, like S100A13, p40 Syt1 is also constitutively released from cells under normal cell culture conditions (LaVallee et al., 1998). Experiments using either an antisense strategy or the expression of a dominant-negative p65 Syt1 mutant, as well as immunoblot analysis of the exported FGF1 complex at non-reducing low denaturation conditions for electrophoresis, demonstrated that, similarly to S100A13, p40 Syt1 is a crucial component of the FGF1 release complex (LaVallee et al., 1998; Tarantini et al., 1998).

Surprisingly, unlike S100A13, p40 Syt1 is dispensable for IL-1α release (Tarantini et al., 2001). However, it is conceivable that IL-1α uses another member of the synaptotagmin family or other C2-domain-containing polypeptides, for example calpain, the intracellular protease responsible for pIL-1α cleavage, which contains a C2 domain (Goll et al., 2003) and associates with annexin II (Barnes and Gomes, 2002). Interestingly, although expression of FGF1 in the presence of S100A13 inhibits the constitutive release of S100A13, it does not affect release of p40 Syt1 (LaVallee et al., 1998).

Annexin II might also be a part of the FGF1 and IL-1α release complexes. This protein exhibits inducible flipping from the inner to the outer surface of the cell membrane (Peterson, 2003), where it functions as a receptor for plasminogen and plasminogen activators (Hajjar et al., 1994; Hajjar et al., 1998). Studies using amlexanox affinity chromatography were able to resolve annexin II in a non-covalent complex with S100A13 (Oyama et al., 1997), and we have recently demonstrated the presence of annexin II in the brain-derived FGF1-containing multiprotein complex (R.S., I.P. and T.M., unpublished). Since annexin II forms heterotetramers with S100A10 (p11) (Kim and Hajjar, 2002), its participation in the multiprotein complexes might rely upon interactions with S100A13. Furthermore, since annexin II

associates with the inner surface of the plasma membrane (Goll et al., 2003) and the assembly of the FGF1 multiprotein complex also occurs near the inner surface of the plasma membrane (Prudovsky et al., 2002), it is possible that annexin II serves as the site of assembly for the non-classical export of these multiprotein complexes. However, more experiments are needed to verify its role in FGF1 and IL-1α release.

The role of Cu^{2+} in FGF1 and IL-1α export

How do the FGF1 and IL-1α release complexes assemble? Association of the members of these multiprotein aggregates might involve Cu^{2+}. FGF1, IL-1α, S100A13 and p40 Syt1 are Cu^{2+}-binding proteins (Engleka and Maciag, 1992; Landriscina et al., 2001b; Mandinova et al., 2003). Also, Cu^{2+} specifically induces formation of FGF1 but not FGF2 homodimers even though two of the three Cys residues present in FGF1 are conserved in FGF2 (Engleka and Maciag, 1992). In addition, several studies have demonstrated angiogenic and pro-inflammatory effects of Cu^{2+} (Brewer, 2001; Gullino, 1983; Hannan and McAuslan, 1982; Raju et al., 1982; Zoli et al., 1998), indicating that Cu^{2+} might participate in the non-classical release of angiogenic and pro-inflammatory polypeptides. The role of Cu^{2+} in mediating the release of FGF1 and IL-1α export has been examined in some detail, and indeed we and others have demonstrated in a cell-free system that Cu^{2+} is able to induce the formation of a complex containing p40Syt1, FGF1 and S100A13 at a molar ratio of 1:2:2, respectively, as well as the formation of a heterotetrameric (2:2) IL-1α-S100A13 complex (Landriscina et al., 2001b; Mandinova et al., 1998). The depletion of intracellular free Cu^{2+} through continuous application of a specific chelator, tetrathiomolybdate (TTM), can attenuate the stress-induced release of IL-1α and FGF1, as well as of S100A13 when co-expressed with IL-1α or FGF1 (Landriscina et al., 2001b; Mandinova et al., 2003). These data indicate that the stress-induced Cu^{2+}-dependent assembly of IL-1α and FGF1 multiprotein release complexes is indeed a prerequisite for the non-classical export of these proteins in vitro (Fig. 2).

The plasma membrane as a platform for the assembly of release complexes

Considerable experimental evidence indicates that the Cu^{2+}-dependent formation of IL-1α and FGF1 multiprotein release complexes occurs at the inner leaflet of the cell membrane. Indeed, TTM treatment does not prevent the stress-induced migration of FGF1 to the cell membrane (Prudovsky et al., 2002), and thus complex formation (including formation of the FGF1 homodimer) does not appear to be important for the intracellular transport of FGF1 to the cell periphery. Moreover, dominant-negative mutants of S100A13 and p40 Syt1 that are known to inhibit FGF1 release are transported to the cell membrane in response to heat shock, and they do not prevent the stress-induced translocation of FGF1 to the periphery (Prudovsky et al., 2002). Apparently, the members of the FGF1 multiprotein complex follow independent stress-induced pathways to the cell periphery. Interestingly, FGF1, IL-1α, p40 Syt1 and members of the S100 family can all bind acidic phospholipids in a cell-free system (Heizmann et al., 1998; Marqueze et al., 2000; Mandinova et al., 2003; Tarantini et al., 1995). Furthermore, mutational analyses have revealed specific acidic phospholipid-binding domains in FGF1 (Tarantini et al., 1995) and Syt1 (Fernandez et al., 2001). Interestingly, a few of these acidic phospholipids are asymmetrically distributed between the leaflets of the plasma membrane (Pomorski et al., 2001) and thus, under normal conditions, acidic phospholipids such as phosphatidylserine localize preferentially to the inner leaflet. However, in response to a variety of different stresses, including heat shock, phosphatidylserine flips to the outer leaflet (Sims and Wiedmer, 2001).

Phosphatidylserine could drive the transmembrane translocation of the IL-1α and FGF1 release complexes since immunofluorescence data suggest that the inner side of the cell membrane is a platform for the assembly of IL-1α and FGF1 release complexes after the participant proteins reach the membrane through heat-shock-induced, actin-dependent transport. Cu^{2+} ions needed for the assembly of release complexes might be provided by transmembrane Cu^{2+} transporters (Finney and O'Halloran, 2003), and it is noteworthy that the recently characterized human Cu^{2+} transporter 1 (hCtr1) is activated by cellular stress (Lee et al., 2002). Since free Cu^{2+} is virtually absent from the cytosol (Rae et al., 1999), the inner leaflet of the cell membrane is the most likely locale for the function of transient Cu^{2+} ions in the assembly of the FGF1 and IL-1α multiprotein complexes. However, it is unclear whether the Cu^{2+} ions involved in the formation of these complexes are released into the extracellular compartment with the exported polypeptides or whether they are recycled back to their intracellular transporters.

Potential role of detergent-like properties and the molten globule state of proteins in facilitating non-classical protein export

The key moment in non-classical export is translocation across the cell membrane. The mechanism might involve local destabilization of the phospholipid bilayer at the inner surface of the plasma membrane, which would allow the protein to insert into the membrane and eventually exit the cell. Several proteins including bactericidal peptides (Wiese et al., 2003) and viral fusion proteins (Dutch et al., 2000) have detergent-like properties that destabilize and permeabilize phospholipid bilayers. It has been observed that FGF1 has similar properties, demonstrating that it can induce temperature-dependent permeabilization of phosphatidylserine/phosphatidylglycerol liposomes (Mach and Middaugh, 1995). IL-1α also behaves similarly (Oku et al., 1995; Mandinova et al., 2003). The phospholipid-binding and detergent-like activities of these proteins indicate that the inner leaflet of the cell membrane could contain sites that recognize the multiprotein complexes destined for release. These sites could contain specific acidic phospholipid 'signatures' that determine both the composition of assembled protein aggregates and the export mechanism.

Translocation of a protein across the bilayer might require conformational changes that increase its hydrophobicity. Proteins can achieve this by adopting a 'molten globule' conformation (Ptitsyn, 1995). This is a partially unfolded intermediate conformation assumed during denaturation and renaturation (Arai and Kuwajima, 2000; Ptitsyn, 1995). It is characterized by (1) the presence of secondary structure, (2)

the absence of most of the tertiary structure normally produced by tight packing of side chains, (3) a relative compactness (a radius of gyration only 10-30% larger than that of the native state), and (4) the presence of a loosely packed hydrophobic core that increases the hydrophobic surface accessible to solvent (Arai and Kuwajima, 2000). The fourth characteristic could allow proteins to traverse lipid bilayers (Bychkova et al., 1988), and it has been reported that FGF1 exhibits a temperature-dependent molten globule conformation (Sanz et al., 2002). Additional studies using two-dimensional nuclear magnetic resonance have confirmed this and underlined the importance of an all-β-barrel structure for formation of the molten globule (Srisailam et al., 2002). This structural feature might therefore enable FGF1, IL-1α and the other polypeptide components of the release complex to lose their solubility in an aqueous environment and simultaneously become soluble in a non-aqueous lipophilic environment, which is a prerequisite for their transport through the plasma membrane. It is interesting to note that the β-barrel structure may be responsible for the ability of FGF1 to form amyloid-like fibrils (Srisailam et al., 2003). However, whether this feature contributes to the Cu^{2+}-induced assembly of the FGF1 (Landriscina et al., 2001b) and IL-1α (Mandinova et al., 2003) high-molecular-weight complexes formed prior to export is not known. Interaction with acidic phospholipids might also significantly contribute to the transition of FGF1 and possibly IL-1α to a molten globule conformation. The importance of unfolded or partially unfolded protein conformations is

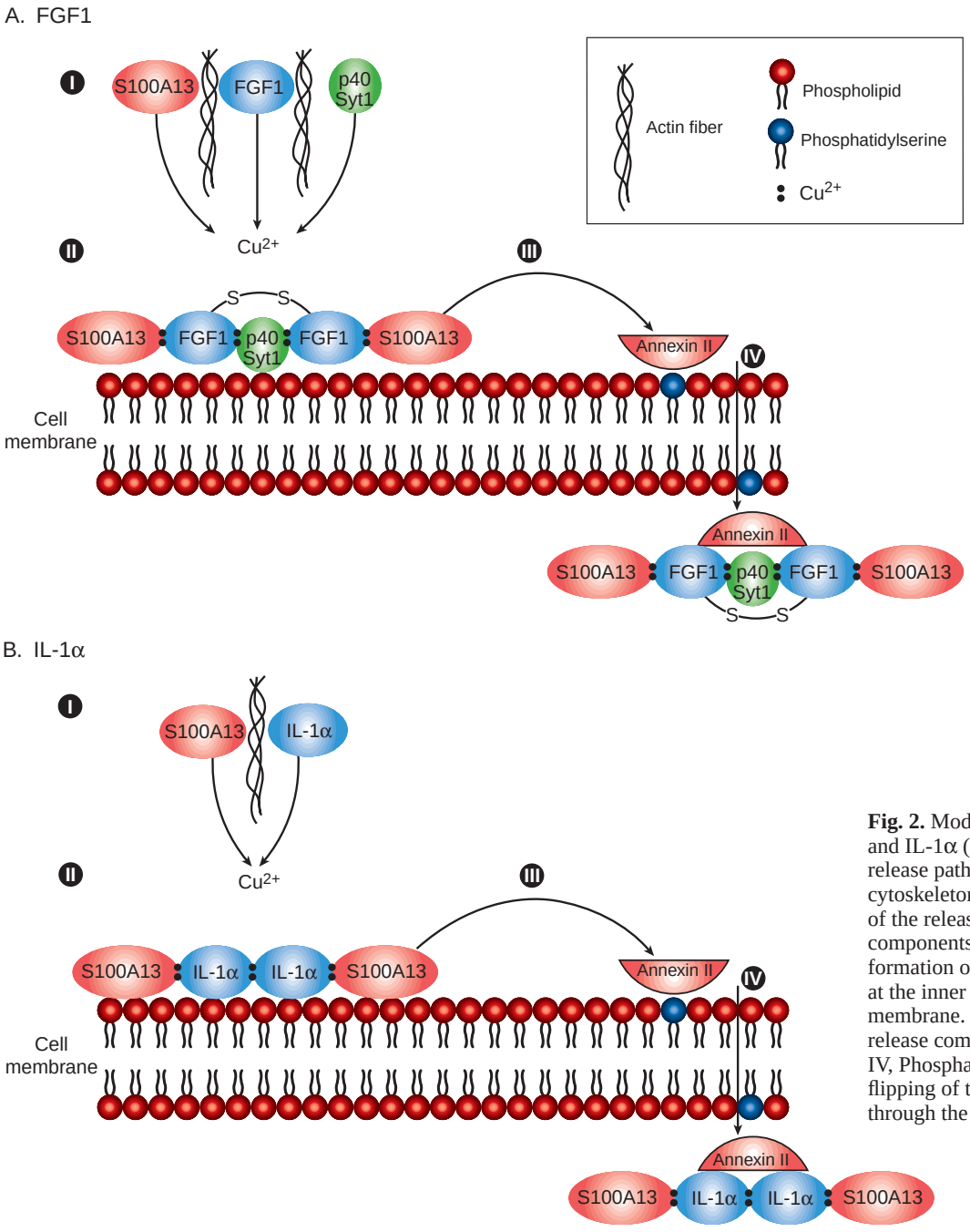

Fig. 2. Models of FGF1 (above) and IL-1α (below) stress-induced release pathways. I, Actin cytoskeleton-dependent transport of the release complex components. II, Cu^{2+}-dependent formation of the release complex at the inner leaflet of the cell membrane. III, Association of the release complex with annexin II. IV, Phosphatidylserine-dependent flipping of the release complex through the cell membrane.

stressed by results demonstrating that, upon overexpression of rhodanese and GFP, excess unfolded proteins are evacuated from the cells through non-classical export pathways (Sloan et al., 1994; Tanudji et al., 2002).

Whereas there is no direct biophysical evidence for the ability of either S100A13 or p40 Syt1 to assume the molten globule conformation, both proteins are known to be constitutively released independently of FGF1 or IL-1α (Landriscina et al., 2001a; LaVallee et al., 1998) and interact with acidic phospholipids. They could therefore play a role as chaperones that stabilize FGF1 and possibly IL-1α in a molten globule conformation. Indeed, observing the interaction between a bacterial pilin and its chaperone, Knight and co-authors (Zavialov et al., 2003) have recently demonstrated that chaperones can maintain polypeptides in a partially folded, high-energy state. Previously, cytosolic chaperones, such as members of the Hsp70 family, had been shown to maintain the mitochondrial pre-proteins in a translocation-competent conformation, which is crucial for their post-translational import into mitochondria (Gordon et al., 2000). It is possible that S100A13 performs a chaperone-like service needed for membrane translocation since its overexpression alleviates the requirements of IL-1α and FGF1 export for new transcription and translation (Landriscina et al., 2001b; Mandinova et al., 2003). Indeed, some other members of the S100 family have chaperone activity (Heizmann et al., 2002), and S100A10, also known as p11, serves as a chaperone for the hepatitis virus B polymerase and is needed for its nuclear translocation (Choi et al., 2003). Also, acting as a chaperone, it appears to be crucial for insertion of annexin II into the plasma membrane, as well as its thrombin-induced flipping to the outer surface of the plasma membrane (Peterson et al., 2003).

The pathological significance of non-classical FGF1 and IL-1α export

The elucidation of the mechanisms responsible for the non-classical export of FGF1 and IL-1α has required the use of in vitro methods of analysis and, as a result, it has been difficult to determine the role of these mechanisms in vivo and/or in pathological processes. However, clinical studies pioneered by G. Brewer and S. Merajver (Brewer et al., 2000; Cox et al., 2001) in which the Cu^{2+} chelator TTM was used to manage the progress of stage IV tumors in humans have provided insight into the potential role of TTM as an angiogenic inhibitor capable of attenuating mammary gland tumor formation in the Her transgenic mouse (Pan et al., 2002). Since these studies suggested that TTM can repress the transcriptional activation of NF-κB, and NF-κB lies downstream of IL-1 receptor signaling (Baldwin, 1996), it appeared possible that TTM functions as a repressor of non-classical IL-1α export. Both FGF1 and IL-1 play a pro-angiogenic role in vivo (Friesel and Maciag, 1999; Voronov et al., 2003) although, in vitro, FGF1 stimulates proliferation and migration of endothelial cells (Maciag et al., 1979; McMahon et al., 1997), whereas IL-1α inhibits both of these activities (Maier et al., 1990). It appears that the regulation of angiogenesis and inflammation involves a coordination of non-classical FGF1 and IL-1α release. Indeed, IL-1α stimulates the infiltration of tissues with macrophages (Dinarello, 1996), which present an abundant source of the FGF prototypes (Sano et. al., 1990; Brogi et al., 1993). The absence of extracellular IL-1α in a tumor setting would limit the recruitment of FGF1-laden mononuclear cells (Sano et al., 1990; Sano et al., 1992) to tumor sites exhibiting an anoxic and/or hypoxic microenvironment. Thus, in the absence of mononuclear cell infiltration, FGF1 would not be delivered to the tumor environment and, even if FGF1 was available within the tumor microvasculature itself, TTM would also repress its export.

Interestingly, a similar mechanism has been proposed to explain the response to injury in large vessels as a result of catheter-mediated clinical management of atherosclerotic arteries (Mandinov et al., 2003). Since the infiltration of mononuclear cells into the injured area in response to the release of IL-1α could result in the generation of an FGF1-rich microenvironment, and FGF1 is a potent mitogen for the vascular smooth muscle cells (Winkles et al., 1987), its export into the extracellular compartment could be responsible for the onset of restenosis. Indeed, the long-term administration of TTM significantly suppresses restenosis induced by catheter injury in the rat carotid artery (Mandinov et al., 2003). The arterial walls of TTM-treated rats display a strong attenuation of neointimal growth, impaired vasa vasorum formation, little, if any, macrophage/monocyte infiltration and, most importantly, very low levels of FGF1 and IL-1α expression when compared with injured arteries from control animals. Thus, the inhibition of restenosis by TTM could be due to the ability of the Cu^{2+} chelator to repress the stress-induced release of pro-inflammatory IL-1α, which would prevent infiltration of mononuclear cells known to be a source of pro-angiogenic and pro-restenotic FGF1 in the wall of the damaged vessel. These data also suggest that the repression of non-classical FGF1 and IL-1α export by Cu^{2+} chelation might ultimately be useful for the clinical management of pro-inflammatory angiogenesis in humans.

These data corroborate the preclinical and clinical reports on the ability of TTM to inhibit solid tumor growth (Brewer et al., 2000; Cox et al., 2001), which depends on the availability of pro-angiogenic polypeptides (Folkman, 2002). Thus, the potential significance of the role of Cu^{2+} as a mediator of the non-classical export of FGF1 and IL-1α could provide an alternative approach for the clinical management of other pathological conditions dependent on pro-inflammatory angiogenesis, such as rheumatoid arthritis (Maini and Taylor, 2000). Indeed, studies have demonstrated that Zn^{2+}/Cu^{2+} chelation can repress the onset of Alzheimer's disease in the β-amyloid transgenic mouse (Cherny et al., 2001). Because the β-amyloid gene is known to be regulated by IL-1α in human endothelial cells (Goldgaber et al., 1989), it is likely that the repression of Alzheimer's disease is due, at least in part, to the absence of extracellular IL-1α. The recent report (Voronov et al., 2003) that IL-1α- and IL-1β-null mice cannot sustain an active angiogenic environment to support tumor growth is consistent with the premise that the function of these signal-peptide-less polypeptides is crucial for the regulation of pro-inflammatory angiogenic responses in vivo.

Future directions

Several important questions related to non-classical polypeptide release remain to be answered. Are there other alternative pathways for non-classical export of FGF1 and IL-

1α and, if so, how are they regulated? Is the molten globule a common feature of such mechanisms? What are the transporter molecules responsible for actin-dependent translocation of the proteins from the cytosol to the cell membranes? How is this translocation induced by cellular stress? Which phospholipids or which groups of phospholipids interact with specific protein members of the release complexes? Do phospholipid signatures permanently exist in the inner leaflet of the cell membrane or are they arranged in response to cellular stress? How does the Cu^{2+}- and phospholipid-dependent formation of multiprotein release complexes induce their subsequent translocation across the cell membrane? What is the source of energy used for stress-induced transmembrane translocation? Solving these problems will result in a better understanding of the non-classical protein release and eventually in an improved ability to regulate both inflammation and angiogenesis.

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