

Unconventional protein secretion: membrane translocation of FGF-2 does not require protein unfolding

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Summary

Endoplasmic reticulum/Golgi-dependent protein secretion depends on signal peptides that mediate membrane translocation of nascent secretory proteins into the lumen of the endoplasmic reticulum. Classical secretory proteins are transported across the membrane of the endoplasmic reticulum in an unfolded conformation, which is similar to protein import into mitochondria. This process is mediated by Sec61, the protein-conducting channel of the endoplasmic reticulum. Employing both FACS-based *in vivo* transport assays and confocal microscopy, we now show that fibroblast growth factor 2 (FGF-2), a pro-angiogenic mediator exported from mammalian cells by an

unconventional secretory pathway, does not need to be unfolded in order to be released into the extracellular space. These findings suggest that the molecular apparatus mediating export of FGF-2 is not only distinct from classical translocation machineries in terms of molecular identity but also operates in a mechanistically distinct manner that allows membrane translocation of FGF-2 in a folded conformation.

Key words: Unconventional protein secretion, Nonclassical export, Fibroblast growth factor, FGF-2, Membrane translocation, Protein targeting

Introduction

Many examples of protein translocation across membranes such as import of classical secretory proteins into the endoplasmic reticulum (ER) (Walter et al., 1984; Rapoport et al., 1996), import of proteins into mitochondria (Schatz, 1996; Neupert, 1997; Pfanner, 2000) and peroxisomes (Subramani et al., 2000) as well as protein import into and export from the nucleus (Görlich and Kutay, 1999) are understood in great detail. In striking contrast, the phenomenon of unconventional protein secretion (also known as nonclassical protein export or ER/Golgi-independent protein secretion) from eukaryotic cells has been known for more than 10 years and yet the molecular mechanism as well as the molecular identity of machinery components involved remain elusive (Cleves, 1997; Hughes, 1999; Nickel, 2003). Intriguingly, several lines of evidence indicate that various kinds of mechanistically distinct nonclassical export routes may exist (Hughes, 1999; Nickel, 2003). The group of unconventionally secreted proteins comprises physiologically crucial factors such as the angiogenic growth factors FGF-1 (fibroblast growth factor 1) and FGF-2 (Rogelj et al., 1989; Mignatti and Rifkin, 1991; Jackson et al., 1992; Mignatti et al., 1992; Florkiewicz et al., 1995; Jackson et al., 1995; Florkiewicz et al., 1998; Trudel et al., 2000; Engling et al., 2002), inflammatory cytokines such as interleukin 1 β (Auron et al., 1987; Rubartelli et al., 1990; Andrei et al., 1999), the galectins, a family of β -galactoside-specific lectins of the extracellular matrix (Cooper and Barondes, 1990; Lindstedt et al., 1993; Sato et al., 1993; Cho

and Cummings, 1995; Cleves et al., 1996; Lutowski et al., 1997; Mehul and Hughes, 1997; Seelenmeyer et al., 2003), viral proteins (Chang et al., 1997; Elliott and O'Hare, 1997; Lecellier et al., 2002; Trotman et al., 2003) and parasitic surface proteins potentially involved in the regulation of host cell infection (Denny et al., 2000). Consistently, unconventional secretory processes have been shown to be gated in most cases (reviewed by Hughes, 1999; Nickel, 2003) – for example, interleukin 1 β secretion is induced on activation of monocytes (Rubartelli et al., 1990; Andrei et al., 1999), secretion of galectin-1 is regulated during differentiation (Cleves et al., 1996; Lutowski et al., 1997) and externalization of FGF-1 is triggered under stress conditions such as heat shock treatment (Jackson et al., 1992; Shin et al., 1996).

Proteins destined for the classical pathway of eukaryotic protein secretion are translocated across the membrane of the ER in an unfolded conformation. In cotranslational transport, soluble secretory proteins typically contain an N-terminal signal peptide that directs the nascent chain in a signal recognition particle (SRP)-dependent manner to the translocation apparatus of the ER (Walter et al., 1984; Rapoport et al., 1996). When protein translation resumes, the growing polypeptide is translocated through the protein conducting channel (Sec61) (Rapoport et al., 1996) in an unfolded conformation. Similarly, proteins imported into mitochondria need to be unfolded in order to get transported across the outer and inner membranes of these organelles (Eilers and Schatz, 1986; Wienhues et al., 1991). When

compared to protein secretion by bacteria or protein import into lysosomes, these examples appear to fit well into a general model of protein transport across membranes in which proteins generally get unfolded before translocation (Schatz and Dobberstein, 1996; Salvador et al., 2000). Classical exceptions to this rule are protein exchange between the nucleus and the cytoplasm (Görlich and Kutay, 1999) and protein import into peroxisomes (Häusler et al., 1996; Purdue and Lazarow, 2001).

In terms of experimental proof, key findings have been made by experiments using mitochondrial reporter proteins that were fused to dihydrofolate reductase (DHFR), an enzyme whose three-dimensional structure can be stabilized by the folate derivative aminopterin (Eilers and Schatz, 1986; Wienhues et al., 1991). Thus, employing the DHFR domain as part of a specific reporter molecule, protein translocation across a membrane can be inhibited in the presence of aminopterin in case protein unfolding is required for this process.

In the current study we made use of the DHFR system to analyse whether the process of unconventional secretion of FGF-2 requires protein unfolding during membrane translocation. For this purpose, we have generated a stable cell line that inducibly expresses FGF-2-GFP (green fluorescent protein) as a fusion protein with DHFR. On the basis of an experimental system that enables us to quantify FGF-2 externalization in living cells (Engling et al., 2002), we report that membrane translocation of FGF-2-GFP-DHFR is fully functional in the presence of aminopterin – that is, under conditions that prevent unfolding of the DHFR domain. As a positive control, using the same cellular background and expression system, we show that membrane translocation of a reporter molecule that is composed of an N-terminal mitochondrial targeting sequence (MTS), GFP and DHFR (MTS-GFP-DHFR) is inhibited in the presence of aminopterin. Our results, therefore, establish that FGF-2 can be exported from mammalian cells in a folded conformation. Thus, the molecular mechanism of membrane translocation of unconventionally secreted proteins differs strikingly from the classical examples of protein transport across membranes such as translocation of secretory proteins into the lumen of the ER or protein import into mitochondria.

Materials and Methods

Antibodies

Affinity-purified anti-GFP antibodies were generated as described previously (Engling et al., 2002). Monoclonal anti-DHFR antibodies were purchased from BD Biosciences. Allophycocyanin-coupled anti-rabbit secondary antibodies used for FACS analyses were obtained from Molecular Probes; monoclonal anti-His-tag antibodies were purchased from Qiagen. Horseradish peroxidase (HRP)-conjugated secondary antibodies for western blotting were from Biorad, and Alexa 546-coupled secondary antibodies used for confocal microscopy came from Molecular Probes.

Generation of model cell lines expressing FGF-2 fusion proteins

All cDNA constructs were based on the retroviral vector pREV-TRE2 (Clontech), which contains a doxycycline/transactivator-responsive element for the initiation of mRNA formation. The open reading frames of FGF-2 (18 kDa isoform) and eGFP originated from the vectors pT7T3D-Pac (I.M.A.G.E. Consortium No. 1690025) and pEGFP1 (Clontech), respectively. The murine DHFR open reading

frame was derived from pQE16 (Qiagen), and the open reading frame of the mitochondrial targeting sequence present in the S9 subunit of the mitochondrial F₀ ATPase was obtained from the vector pYES-mtGFP (Westermann and Neupert, 2000). In case of the DHFR ORF the serine residue at position 7 (pQE16) was mutated to a cysteine residue to restore the murine DHFR wild-type sequence. On the basis of these sequence modules, the fusion constructs FGF-2-GFP-DHFR and MTS-GFP-DHFR (Fig. 1A) were generated and ligated into pREV-TRE2, which was subsequently used for the production of retroviral particles carrying these reporter molecules (Engling et al., 2002). Three days after retroviral transduction of CHO_{MCAT-TAM2} cells (Engling et al., 2002), including 12 hours of incubation in the presence of 1 µg/ml doxycycline (Sigma), 50,000 cells from each transduction sample were isolated by FACS sorting based on GFP-derived fluorescence. The two pools of cells were incubated for 7 days at 37°C in the absence of doxycycline followed by the isolation of 50,000 cells from each population that did not display GFP-derived fluorescence at this point. Each population was then cultured for another 7 days at 37°C, including 12 hours in the presence of 1 µg/ml doxycycline at the end of this procedure. Single cells were then isolated by FACS sorting based on GFP-derived fluorescence. These clones were propagated and used for the preparation of frozen stocks. The newly generated clonal cell lines were termed CHO_{FGF-2-GFP-DHFR} and CHO_{MTS-GFP-DHFR}, respectively, in order to reflect the reporter molecule expressed. CHO_{FGF-2-GFP} cells used in this study have been generated according to the same protocol as described previously (Engling et al., 2002).

Biochemical characterization of the cell lines and reporter molecules

To analyse the expression of the various reporter molecules, cells grown on culture dishes were washed with PBS followed by cell detachment using cell dissociation buffer (Life Technologies). The cells were collected by low speed centrifugation and resuspended in SDS sample buffer. After SDS PAGE and western blotting the reporter molecules were detected with anti-GFP antibodies.

To biochemically characterize the stability of the DHFR domain in the presence or absence of aminopterin, FGF-2-GFP-DHFR- and MTS-GFP-DHFR-expressing cells were lysed in a detergent-containing buffer (100 mM HEPES-KOH (pH 7.4), 2 mM CaCl₂, 0.2% Triton X-100). The samples were incubated for 15 minutes at room temperature in the absence or presence of 50 µM aminopterin followed by the addition of trypsin (200 µg/ml). After 30 minutes of incubation at 4°C, the reaction was terminated by adding protease inhibitors. Samples were separated by SDS PAGE and analysed by western blotting using monoclonal anti-DHFR antibodies.

Confocal microscopy

CHO_{FGF-2-GFP}, CHO_{FGF-2-GFP-DHFR} and CHO_{MTS-GFP-DHFR} cells were grown on glass coverslips for 18 hours at 37°C in the presence of 1 µg/ml doxycycline. The cells were then processed, including paraformaldehyde fixation (3% w/v, 20 minutes at 4°C) without permeabilization followed by antibody processing as indicated. Alexa546-coupled secondary antibodies were used for cell-surface staining experiments. The specimens were mounted in Fluoromount G (Southern Biotechnology Associates) and viewed with a Zeiss LSM 510 confocal microscope.

Fluorescence activated cell sorting (FACS)

CHO_{FGF-2-GFP}, CHO_{FGF-2-GFP-DHFR} and CHO_{MTS-GFP-DHFR} cells were grown under the conditions indicated in the corresponding figure legends. To detach the cells from the culture plates without using protease-based protocols, cell dissociation buffer (Life Technologies) was used to generate a cell suspension devoid of cell aggregates.

Where indicated, cells were treated with antibodies for 1 hour at 4°C on a rotating wheel. Wash procedures were carried out by sedimenting the cells at 200 *g* for 2 minutes at 4°C. Before the FACS analysis, propidium iodide (1 µg/ml) was added in order to detect damaged cells.

GFP- and allophycocyanin-derived fluorescence were analysed using a Becton Dickinson FACSCalibur flow cytometer. Autofluorescence was determined by measuring noninduced cells which were not treated with allophycocyanin-coupled secondary antibodies. GFP-derived fluorescence and allophycocyanin-derived fluorescence were measured simultaneously without compensation.

Results

Generation of model cell lines designed to study protein folding during membrane translocation of FGF-2

To analyse whether membrane translocation during unconventional secretion of FGF-2 requires protein unfolding, we have generated stable CHO cell lines that express FGF-2-GFP as a fusion protein with dihydrofolate reductase (DHFR) and a C-terminal His-tag (Fig. 1A). This experimental system enables us to control the folding state of the reporter molecule in living cells as the membrane-permeable DHFR ligand aminopterin can be used to stabilize the tertiary structure of the DHFR domain (Eilers and Schatz, 1986; Wienhues et al., 1991). The key aim of this study was to analyse FGF-2 export under conditions that do not allow unfolding of the reporter molecule, and for this purpose we used a FACS-based export assay introduced previously (Engling et al., 2002). As a positive control, we generated a stable CHO cell line that expresses a fusion protein composed of an N-terminal mitochondrial targeting sequence (derived from the S9 subunit of the mitochondrial F₀ ATPase) (Westermann and Neupert, 2000), GFP and DHFR/His at its C-terminus (MTS-GFP-DHFR; Fig. 1A).

All reporter constructs were expressed in the same cellular background (CHO_{MCA1-TAM2}) (Engling et al., 2002) and were based on a doxycycline-dependent transactivator system. As shown in Fig. 1B, the various reporter molecules are immunodetectable in cell lysates following the induction of protein expression using doxycycline. In the case of MTS-GFP-DHFR, two low molecular weight forms can be observed in addition to the full-length protein, indicating that the fusion protein was imported into the mitochondrial matrix where the N-terminal domain is proteolytically processed at two independent sites (Ungermann et al., 1994). The main product is cleaved at both sites, whereas the less abundant processing product has been cleaved only once. These results establish the efficient import of MTS-GFP-DHFR into the mitochondrial matrix.

Characterization of model cell lines using confocal microscopy

The clonal cell lines described in Fig. 1 were characterized with regard to the subcellular localization of the various reporter molecules. As shown in Fig. 2, experiments based on confocal microscopy confirmed that the expression of all reporter molecules is strictly dependent on the addition of doxycycline (compare A, D and G (– doxycycline) with B, E and H (+ doxycycline)). In the case of FGF-2-GFP, the protein was found to be distributed between the cytoplasm and the

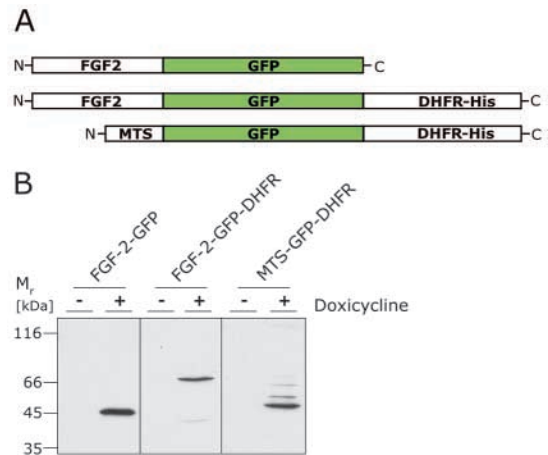


Fig. 1. Generation of model cell lines designed to study protein unfolding aspects during unconventional secretion of FGF-2. (A) cDNA constructs used in this study. CHO_{MCA1-TAM2} cells (Engling et al., 2002) were transduced with retroviral particles carrying the cDNA constructs shown. Clonal cell lines were obtained by FACS sorting. For details, see Materials and Methods. (B) Western blot analysis of model cell lines grown in the absence or presence of doxycycline. Cell lysates were separated on 10% SDS gels followed by protein transfer to a PVDF membrane. The constructs depicted in A were detected using affinity-purified anti-GFP antibodies (Engling et al., 2002). In the case of FGF-2-GFP, the material derived from about 10,000 cells was loaded; in the case of FGF-2-GFP-DHFR and MTS-GFP-DHFR, the material derived from about 20,000 cells was loaded.

nucleus (Fig. 2B). Moreover, secreted FGF-2-GFP could readily be detected on the cell surface as shown by anti-GFP staining of nonpermeabilized cells (Fig. 2C). These results are consistent with data reported previously (Engling et al., 2002). When the FGF-2-GFP-DHFR cell line was analysed by confocal microscopy, a similar intracellular distribution between the cytoplasm and the nucleus was observed (Fig. 2, compare B and E). Like FGF-2-GFP, FGF-2-GFP-DHFR was readily detectable on the cell surface (Fig. 2F), showing that the addition of the DHFR domain to the C-terminus of FGF-2-GFP does not interfere with the export pathway of this reporter molecule. When the FGF-2 domain was replaced by a mitochondrial targeting sequence, the reporter molecule (MTS-GFP-DHFR) was found in punctate structures distributed throughout the cytoplasm (Fig. 2H). In combination with the biochemical data showing matrix-specific proteolytical processing of the reporter (Fig. 1B), we conclude that MTS-GFP-DHFR is efficiently translocated into mitochondria. As expected, no surface staining could be observed when nonpermeabilized MTS-GFP-DHFR-expressing cells were processed with anti-GFP antibodies (Fig. 2I).

Quantitative analysis of unconventional secretion of FGF-2-GFP and FGF-2-GFP-DHFR fusion proteins in living cells based on FACS

To compare the externalization of FGF-2-GFP versus FGF-2-GFP-DHFR on a quantitative basis in living cells, we used a FACS-based reconstitution system introduced previously (Engling et al., 2002). Similar to the experiments shown in Fig.

2, this assay makes use of the ability of externalized FGF-2 to bind to cell-surface heparan sulfate proteoglycans. Thus, extracellular FGF-2 can be decorated with specific antibodies in order to quantify the exported population based on FACS. As the FGF-2-GFP-DHFR fusion protein contains a C-terminal His-tag, we used both anti-GFP and anti-His-tag antibodies to detect secreted FGF-2 fusion proteins. In all cases, GFP-derived fluorescence was measured simultaneously to compare the expression levels of the various reporter molecules. As shown in Fig. 3, the expression of all reporter molecules strictly depends on the addition of doxycycline (grey versus green curve). When anti-GFP and anti-His-tag antibodies were compared in the case of FGF-2-GFP (Fig. 3A,B), only anti-GFP antibodies provided a cell-surface signal. Similarly, irrespective of the use of anti-GFP or anti-His-tag antibodies, no cell-surface signal could be observed when noninduced cells were analysed (Fig. 3A,B; grey curves), thereby illustrating the specificity of the antibodies used. The expression level of FGF-2-GFP-DHFR was found to be slightly lower than the one of FGF-2-GFP (compare A and C; green curves). However, the FGF-2-GFP-DHFR cell-surface signal (Fig. 3C) was found to be similar to the one of FGF-2-GFP (Fig. 3A). As expected, cell-surface FGF-2-GFP-DHFR could be detected with both anti-GFP- and anti-His-tag antibodies (see above) with similar signal intensities (Fig. 3, compare C and D). On the basis of GFP-derived fluorescence, MTS-GFP-DHFR was found to be expressed at similar levels compared to FGF-2-GFP and FGF-2-GFP-DHFR (Fig. 3, compare A, C

and E). However, as expected, no cell-surface signal was observed in the case of MTS-GFP-DHFR, irrespective of the use of anti-GFP- or anti-His-tag antibodies (Fig. 3E,F). These experiments illustrate the specific export of FGF-2-GFP and FGF-2-GFP-DHFR at similar levels, whereas MTS-GFP-DHFR remains intracellular.

MTS-GFP-DHFR import into mitochondria is inhibited in the presence of aminopterin

To be able to analyse a potential need for protein unfolding during membrane translocation of FGF-2, we first established a positive control that shows a block of protein translocation across a membrane in living cells under conditions preventing protein unfolding. For this purpose, we generated a stable cell line expressing a fusion protein composed of an N-terminal mitochondrial targeting sequence, GFP and a C-terminal DHFR domain (MTS-GFP-DHFR), as introduced in Figs. 1-3. To monitor import of MTS-GFP-DHFR into mitochondria in the absence or presence of aminopterin, we conducted experiments based on both biochemical methods and confocal microscopy. As already shown in Fig. 1B and Fig. 2G-I, MTS-GFP-DHFR is efficiently transported into the mitochondrial matrix. When cells were incubated in the presence of both doxycycline and aminopterin, MTS-GFP-DHFR was found to reside in the cytoplasm and in the nucleus, suggesting that MTS-GFP-DHFR was no longer capable of entering mitochondria (Fig. 4, compare B and C). The partial nuclear

localization of MTS-GFP-DHFR is likely to be caused by the GFP domain of the reporter molecule under conditions where it is not efficiently segregated into mitochondria. These results were confirmed by a biochemical analysis of cell lysates following incubation of cells in the presence or absence of aminopterin. As shown in Fig. 4D, three MTS-GFP-DHFR species (labelled with *, \blacklozenge and \blacktriangle) were detectable using an anti-GFP antibody. The slow-migrating form represents the unprocessed precursor (*), the intermediate form (\blacklozenge) has been cleaved once and the fast-migrating form (\blacktriangle) represents the fully processed protein that is cleaved at two

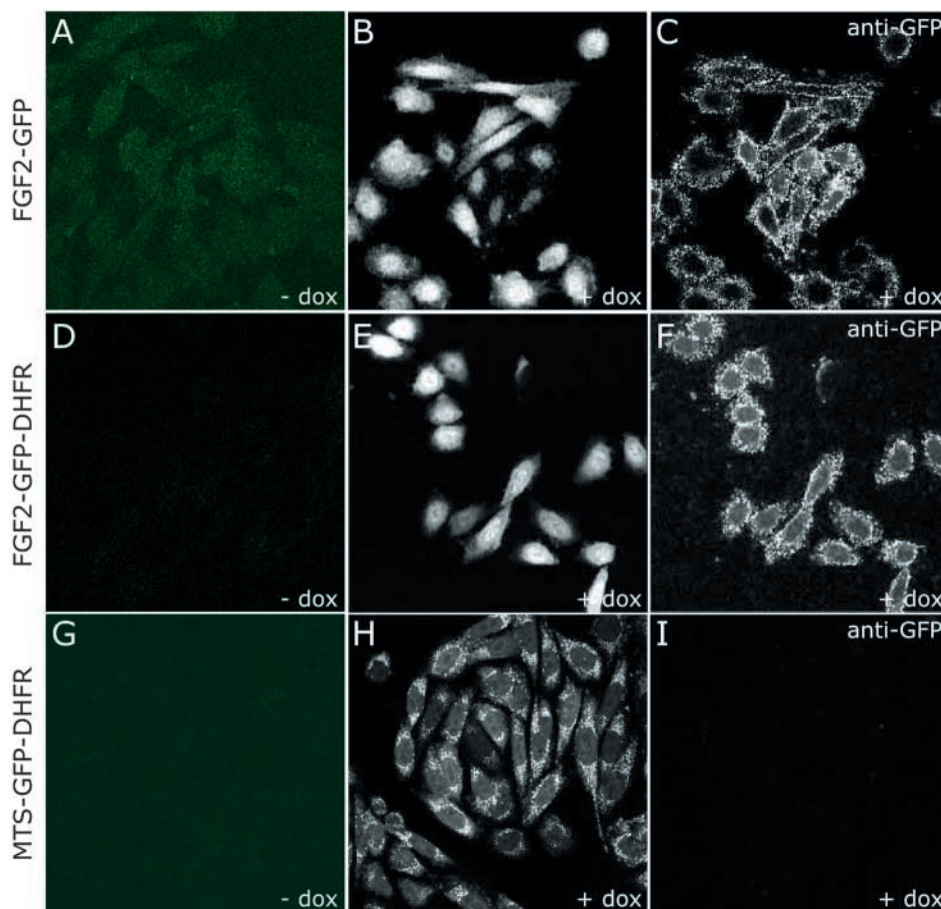


Fig. 2. Characterization of the various model cell lines based on confocal microscopy. CHO_{FGF-2-GFP} (A-C), CHO_{FGF-2-GFP-DHFR} (D-F) and CHO_{MTS-GFP-DHFR} cells (G-I) were grown on coverslips in the absence (panels A,D,G) or presence of doxycycline (panels B,C,E,F,H,I). Cells were fixed without permeabilization using paraformaldehyde followed by immunostaining using affinity-purified anti-GFP antibodies. In B, E and H total GFP fluorescence is shown, whereas in panels C, F and I cell-surface staining is shown.

sites within the mitochondrial matrix. In the absence of aminopterin (Fig. 4D) the fully processed form of MTS-GFP-DHFR (\blacktriangle) prevails, indicating efficient import into the mitochondrial matrix. However, under steady-state conditions, both the precursor and the intermediate form, which is likely to be associated with the mitochondrial membranes, are detectable. In the presence of aminopterin, the amount of the fully processed form of MTS-GFP-DHFR

is largely reduced, illustrating inhibition of protein translocation into the matrix (\blacktriangle). Interestingly, the intermediate form (\blacklozenge) remains unchanged, suggesting that initial insertion and the first proteolytical processing step occur independently of the presence or absence of aminopterin. Strikingly, the precursor and, therefore, the overall amount of MTS-GFP-DHFR species is largely reduced in the presence of aminopterin, suggesting that membrane-arrested MTS-GFP-DHFR is subjected to protein degradation in order to keep the import machinery in a functional state. These findings are consistent with earlier reports showing that mitochondrial import requires protein unfolding before membrane translocation (Eilers and Schatz, 1986; Wienhues et al., 1991).

To use the DHFR domain in the background of the FGF-2 reporter molecule, it was important to show that the DHFR domain of FGF-2-GFP-DHFR actually binds aminopterin and is stabilized by the ligand. For this purpose, we conducted protease-protection experiments in the absence or presence of aminopterin (Fig. 5). Lysates were obtained from doxycycline-induced $CHO_{\text{FGF-2-GFP-DHFR}}$ and $CHO_{\text{MTS-GFP-DHFR}}$ cells. The samples were incubated with trypsin and aminopterin as indicated. In the absence of aminopterin, both MTS-GFP-DHFR (lane 2) and FGF-2-GFP-DHFR (lane 5) were efficiently degraded. However, in the presence of aminopterin, both reporter molecules (lanes 3 and 6, respectively) were significantly stabilized, as indicated by the detection of the DHFR fragment. These findings show that aminopterin binds to FGF-2-GFP-DHFR and stabilizes its DHFR domain, as indicated by the resistance to protease treatment (Fig. 5).

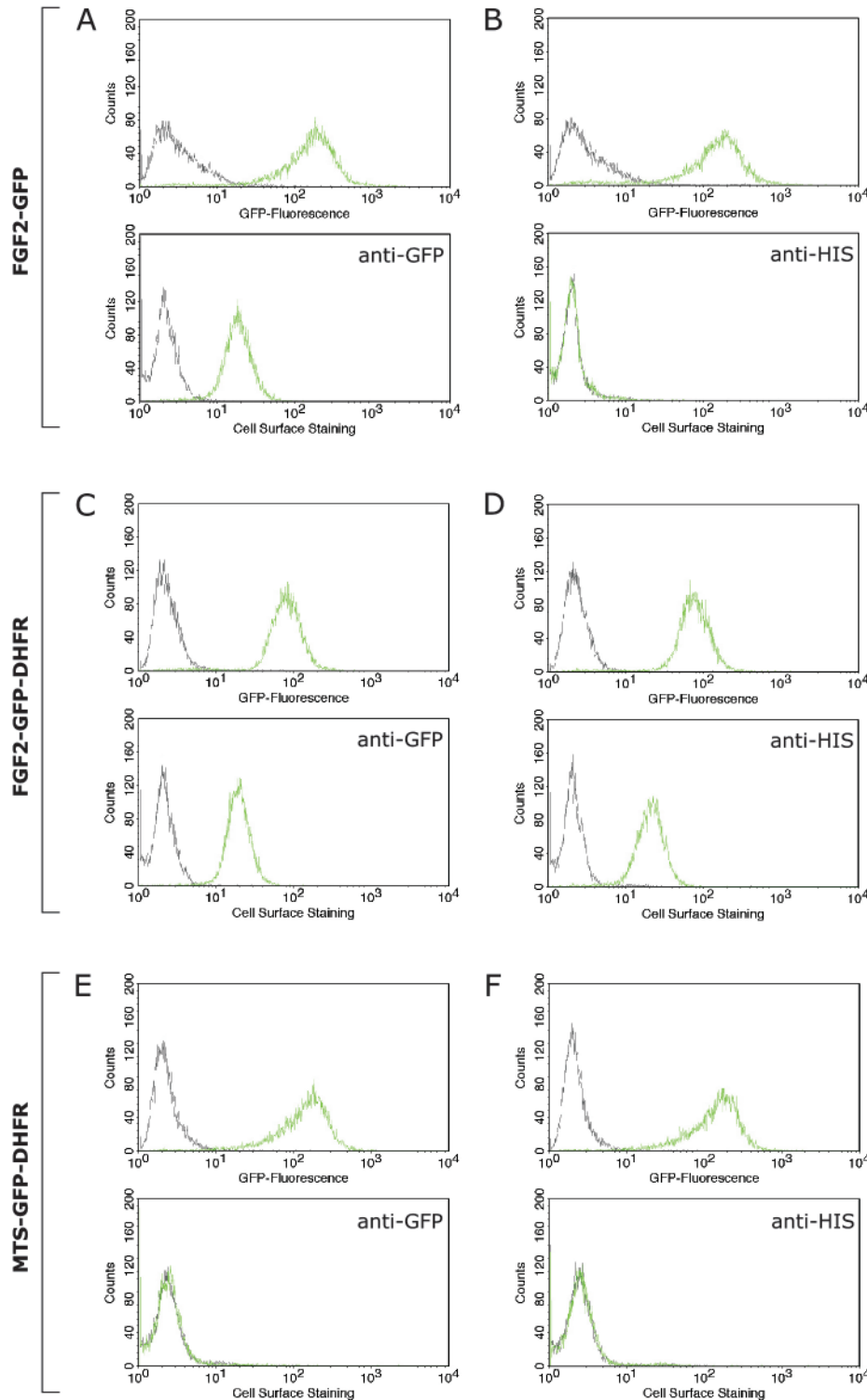


Fig. 3. Quantitative analysis of unconventional secretion of FGF-2-GFP and FGF-2-GFP-DHFR fusion proteins in living cells based on FACS. $CHO_{\text{FGF-GFP}}$ (A,B), $CHO_{\text{FGF-2-GFP-DHFR}}$ (C,D) and $CHO_{\text{MTS-GFP-DHFR}}$ cells (E,F) were grown in the absence (grey curves) or presence (green curves) of doxycycline. Cells were dissociated from the culture plates using a protease-free protocol followed by immunostaining using either affinity-purified anti-GFP (A,C,E) or monoclonal anti-His-tag antibodies (B,D,F). Living cells were analysed simultaneously for both total GFP fluorescence and cell-surface staining employing a BD FACSCalibur system.

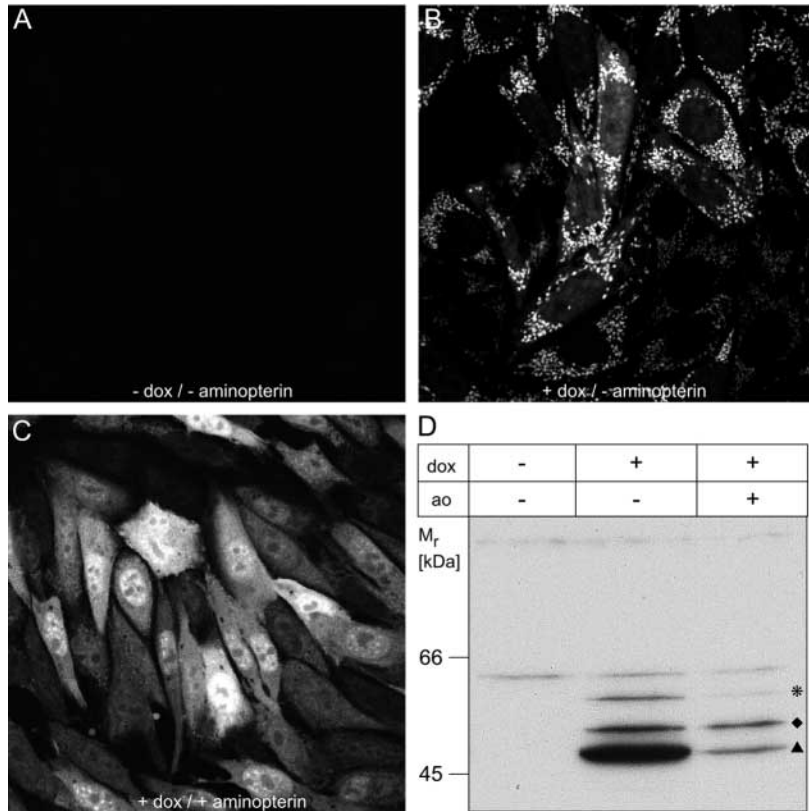


Fig. 4. MTS-GFP-DHFR import into mitochondria is inhibited in the presence of aminopterin. (A-C) CHO_{MTS-GFP-DHFR} cells were grown on coverslips in the absence (A) or presence (B and C) of doxycycline. Cells shown in C were treated with aminopterin (50 mM) throughout induction of protein synthesis. Cells were fixed using paraformaldehyde followed by the analysis of GFP-derived fluorescence using a Zeiss LSM 510 confocal microscope. (D) Biochemical analysis of MTS-GFP-DHFR import into mitochondria in the presence and absence of aminopterin. CHO_{MTS-GFP-DHFR} cells were grown in the presence of doxycycline. Where indicated, aminopterin (ao; 50 mM) was added throughout induction of protein synthesis. The various cell lysates were analysed by SDS-PAGE and western blotting using affinity-purified anti-GFP antibodies. Three species of MTS-GFP-DHFR are detected, the precursor (*) as well as an intermediate (◆) and the fully processed form (▲).

Qualitative analysis of FGF-2 export in the presence of aminopterin based on confocal microscopy

As shown in Fig. 2F, the secreted population of FGF-2-GFP-DHFR can be detected by confocal microscopy as, following export, the FGF-2 domain specifically binds to cell-surface heparan sulfate proteoglycans (Engling et al., 2002). To analyse a potential effect of aminopterin on the export mechanism of this construct, we first conducted qualitative experiments based on confocal microscopy. As shown in Fig. 6D, the presence of aminopterin does not disrupt FGF-2-GFP-DHFR cell-surface localization when compared with controls incubated in the absence of aminopterin (Fig. 6B). In these experiments, FGF-2-GFP-DHFR was detected with anti-GFP antibodies, showing that at least the N-terminal part consisting of the FGF-2 and GFP domains is localized outside the cell. Formally, these data do not rule out that, under these

conditions, the C-terminal part remains inside the cell due to a block of DHFR unfolding in the presence of aminopterin. To establish that the reporter molecule gets exported completely under conditions that do not allow DHFR unfolding, we analysed whether exported FGF-2-GFP-DHFR is accessible for antibodies directed against the C-terminal His-tag of this construct. As shown in Fig. 6F, both the N-terminal part and the C-terminal part of FGF-2-GFP-DHFR are detectable on the cell surface in the presence of aminopterin, suggesting that protein unfolding is not required for membrane translocation of FGF-2.

Quantitative analysis of FGF-2 export in the presence of aminopterin based on FACS

The FACS-based FGF-2 export assay described in Fig. 3 was then used to quantitatively evaluate the results shown in Fig. 6. For this purpose, externalization of FGF-2-GFP-DHFR was measured either in the absence or in the presence of aminopterin employing both anti-GFP and anti-His antibodies directed against the extreme C-terminus. This analysis revealed that the FGF-2-GFP-DHFR expression level increased about threefold in the presence of aminopterin (Fig. 7A; compare a,b with e,f). This effect is probably due to an aminopterin-

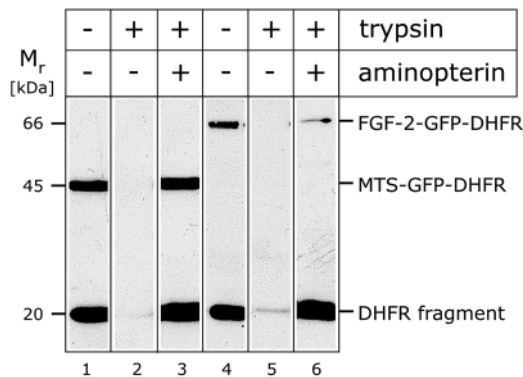


Fig. 5. Stabilization of the DHFR domain of MTS-GFP-DHFR and FGF-2-GFP-DHFR in the presence of aminopterin. Doxycycline-induced CHO_{MTS-GFP-DHFR} cells and CHO_{FGF-2-GFP-DHFR} cells were lysed in a detergent-containing buffer. The lysates were cleared by centrifugation and subjected to protease treatment (200 mg trypsin/ml) in the presence or absence of aminopterin (50 mM). Lanes 1-3: MTS-GFP-DHFR; lanes 4-6: FGF-2-GFP-DHFR. The various samples were subjected to SDS-PAGE and western blotting followed by antigen detection using monoclonal anti-DHFR antibodies. As can be seen in lanes 1 (MTS-GFP-DHFR) and 4 (FGF-2-GFP-DHFR), post-lysis degradation results in the appearance of the DHFR fragment without adding exogenous protease.

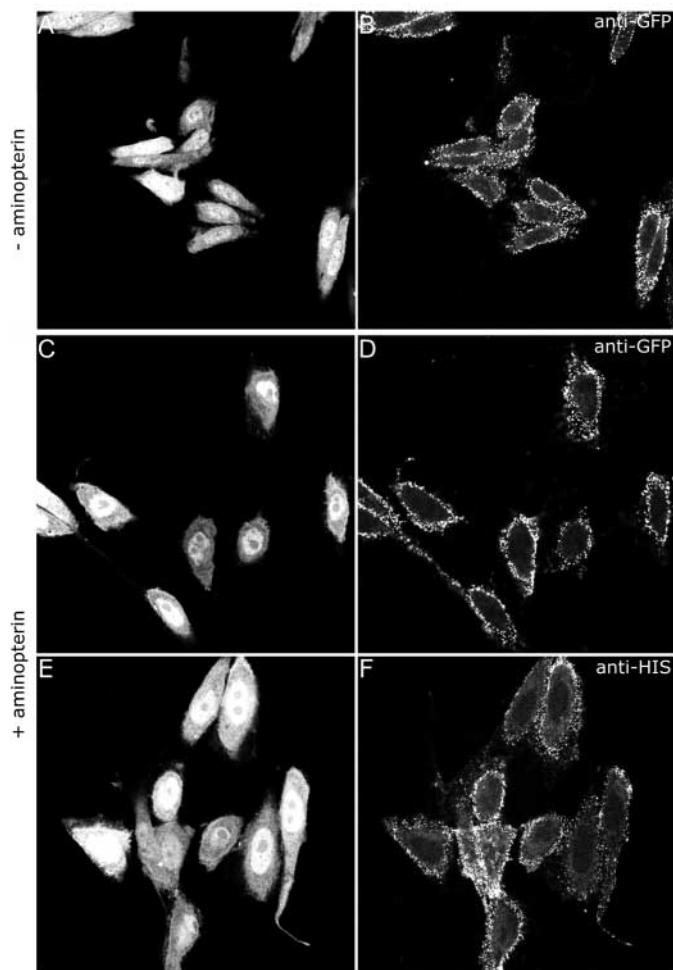


Fig. 6. Qualitative analysis of FGF-2 export in the absence and presence of aminopterin. Doxycycline-induced CHO_{FGF-2-GFP-DHFR} cells were grown on coverslips in the absence (A,B) or presence (C-F) of aminopterin. Cells were fixed without permeabilization using paraformaldehyde followed by immunostaining using either affinity-purified anti-GFP antibodies (B,D) or monoclonal anti-His-tag antibodies (F). Total GFP fluorescence (A,C,E) as well as cell-surface staining (B,D,F) were analysed using a Zeiss LSM 510 confocal microscope.

dependent stabilization of the DHFR domain which might increase the half-life of the reporter molecule. Accordingly, the absolute amounts of FGF-2-GFP-DHFR exported to the cell surface were also found to be increased in the presence of aminopterin, however, in this case only by a factor of about 2 (Fig. 7A; c,d versus g,h). This observation suggests that the secretory apparatus mediating FGF-2 export is saturated with substrate in the presence of aminopterin.

To directly compare the cell-surface signals of FGF-2-GFP-DHFR, we titrated down the amount of doxycycline used to induce protein expression in the presence of aminopterin in order to establish conditions where the expression level of FGF-2-GFP-DHFR was similar in the presence or absence of aminopterin. We used a statistical analysis of three independent experiments and found that FGF-2-GFP-DHFR export to the cell surface is not affected by aminopterin when based on measurements using anti-GFP antibodies (Fig. 7B). When anti-

His antibodies were used to analyse FGF-2-GFP-DHFR export, cell-surface staining was found to actually increase in the presence of aminopterin (Fig. 7B). This effect is probably due to an increase in accessibility of the C-terminal His epitope when aminopterin is bound to the DHFR domain. In any case, these results show that FGF-2-GFP-DHFR membrane translocation is complete in the presence of aminopterin, as both the N-terminal part and the extreme C-terminus of the reporter molecule are accessible on the cell surface.

Discussion

In the current study we addressed the question as to whether membrane translocation in the overall process of FGF-2 externalization requires protein unfolding. For this purpose we combined a FACS-based *in vivo* system reconstituting FGF-2 export (Engling et al., 2002) with a modified FGF-2-GFP reporter molecule that contains the DHFR domain at its C-terminus. The membrane-permeable folate analogue aminopterin (Eilers and Schatz, 1986; Wienhues et al., 1991) was used to stabilize the DHFR domain *in vivo*, which allows us to analyse a potential need for protein unfolding during FGF-2 export. The principal finding of this study is that membrane translocation of FGF-2 by living cells apparently occurs in a folded conformation as an FGF-2-GFP-DHFR fusion protein is efficiently exported in the presence of aminopterin. Importantly, on the basis of antibodies directed against the N-terminal part (anti-GFP) of the reporter, as well as antibodies directed against the extreme C-terminus (anti-His), we showed complete translocation of FGF-2-GFP-DHFR in the presence of aminopterin. As a positive control, based on the same cellular background and expression system, we have generated a reporter molecule that contains a targeting motif for import into mitochondria. In this case, membrane translocation is almost completely inhibited in the presence of aminopterin. Strikingly, the precursor and, therefore, the overall amount of MTS-GFP-DHFR species, is largely reduced in the presence of aminopterin, suggesting that membrane-arrested MTS-GFP-DHFR is subjected to protein degradation in order to keep the import machinery in a functional state. Moreover, we have shown that the DHFR domain fused to either FGF-2-GFP or MTS-GFP is actually stabilized by aminopterin, showing that this drug not only binds to the reporter molecule but also prevents unfolding as shown by protease protection experiments. These findings illustrate the functionality of the DHFR/aminopterin tool in our experimental background and are consistent with earlier reports showing that mitochondrial import requires protein unfolding before membrane translocation (Eilers and Schatz, 1986; Wienhues et al., 1991).

Because all secretory proteins exported by unconventional means are characterized by a relatively small size (Cleves, 1997; Hughes, 1999; Nickel, 2003), it might be conceivable that these proteins do not need to be unfolded during membrane translocation. However, the use of larger fusion proteins to reconstitute FGF-2 export as described here, as well as in other studies (Florkiewicz et al., 1995; Engling et al., 2002), does not pose any problems, i.e. the FGF-2-GFP-DHFR reporter molecule (70 kDa), despite being approximately four times the size of FGF-2 (18 kDa isoform), is secreted almost as efficiently as unmodified FGF-2. A simple explanation for

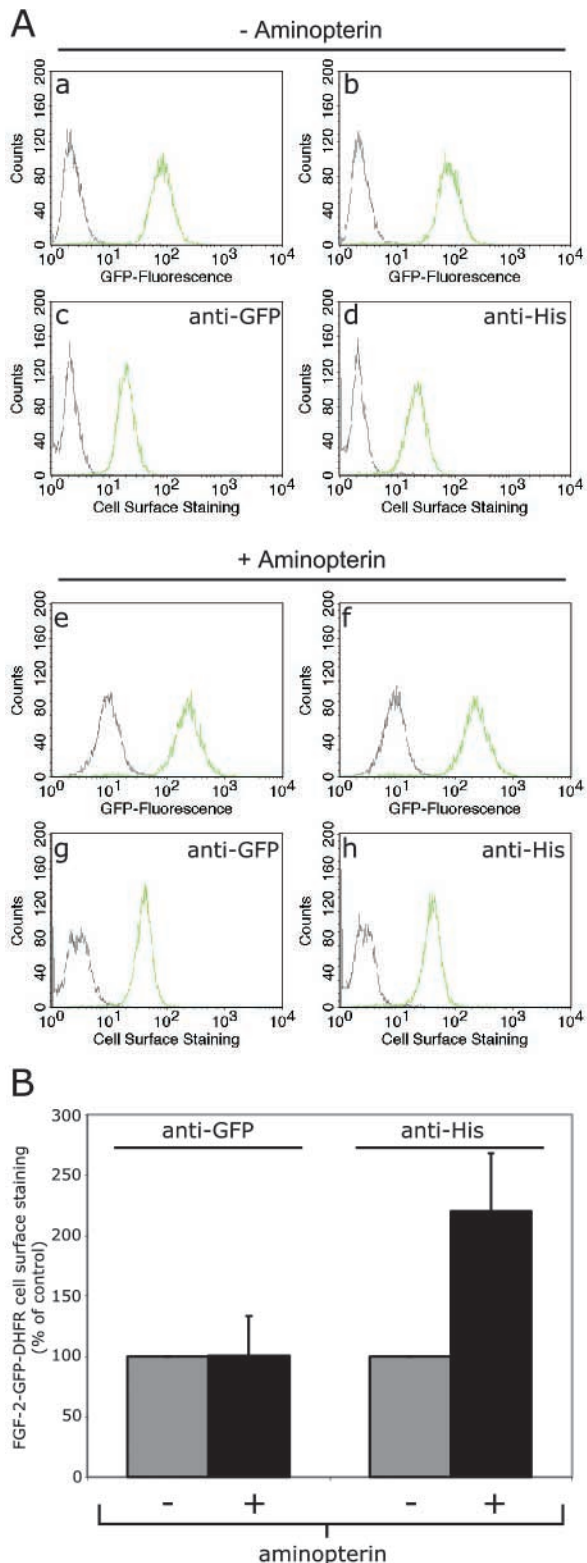


Fig. 7. Quantitative analysis of FGF-2 export in the absence or presence of aminopterin employing FACS. (A) Noninduced (grey curves) and doxycycline-induced (green curves) CHO_{FGF-2-GFP-DHFR} cells were grown in the absence (a-d) or presence (e-h) of aminopterin. Following detachment cells were processed for FACS analysis using either affinity-purified anti-GFP antibodies (a,c,e,g) or monoclonal anti-His-tag antibodies (b,d,f,h). Total GFP fluorescence (a,b,e,f), as well as cell-surface staining (c,d,g,h), were measured simultaneously using a Becton Dickinson FACSCalibur system. (B) Statistical analysis of FGF-2-GFP-DHFR export in the absence and presence of aminopterin based on expression-corrected cell-surface staining. Because aminopterin causes a significant increase of FGF-2-GFP-DHFR expression, the amount of doxycycline added to aminopterin-treated cells was titrated until an expression level was reached similar to that of cells grown in the absence of aminopterin. Under these conditions, FGF-2-GFP-DHFR export was determined on the basis of cell-surface staining. Cell-surface staining measured in the absence of aminopterin was set to 100% and compared with cell-surface staining in the presence of aminopterin ($n=3$).

On the basis of an experimental system functionally reconstituting galectin-1 (Gal-1) export from living cells (Seelenmeyer et al., 2003), we have adapted the experimental strategy described in this study to analyse whether Gal-1 externalization requires protein unfolding. Again, the addition of aminopterin to cells expressing a Gal-1-GFP-DHFR fusion protein did not interfere with secretion (data not shown). Thus, unconventional secretion processes might generally be mediated by machineries that allow membrane translocation of folded cargo molecules. Interestingly, membrane translocation of proteins without the need for protein unfolding is not unprecedented as it has been shown that protein import into glycosomes, a specialized form of peroxisomes found in trypanosomes, apparently occurs in a folded conformation (Häusler et al., 1996; Purdue and Lazarow, 2001; Titorenko et al., 2002). In any case, this study establishes that the molecular machinery mediating nonclassical export of secretory proteins is not only distinct from the Sec61-dependent translocation apparatus of the ER in terms of molecular identity but also operates in a mechanistically distinct manner that allows membrane translocation of FGF-2 in a folded conformation.

Our findings provide the first insight into the molecular mechanism of FGF-2 and Gal-1 export. For example, it has been widely speculated that ABC transporters might mediate unconventional secretory processes in mammalian cells (Kuchler and Thorner, 1992). The rationale for this hypothesis is the coexistence of ABC transporter-based export pathways along with signal-peptide-dependent secretory pathways in bacteria, the latter one being related to the ER-resident translocon of mammalian cells (Schatz and Dobberstein, 1996). The haemolysin transporter HlyB, an integral membrane protein involved in the secretion of the 110 kDa bacterial toxin haemolysin (Blight and Holland, 1990; Holland et al., 1990), is the classical example of bacterial protein secretion mediated by an ABC protein. Recent studies suggest that protein translocation mediated by bacterial ABC transporters requires protein unfolding (Debarbieux and Wandersman, 2001; Sharff et al., 2001). For example, SecB, a bacterial chaperone that keeps cytoplasmic protein precursors in an unfolded state, is required for ABC transporter-dependent secretion of the bacterial protein HasA

this observation would be that FGF-2 gets unfolded during translocation and, therefore, size does not matter. However, our findings do not support this view, suggesting that the molecular mechanism of FGF-2 export differs strikingly from the classical examples of membrane translocation such as protein import into the endoplasmic reticulum or mitochondria.

(Debarbieux and Wandersman, 2001). It has been reported that ABC1 transporters might be involved in the overall process of unconventional secretion of interleukin 1 β secretion by mammalian cells (Hamon et al., 1997; Zhou et al., 2002); however, this conclusion is based solely on pharmacological evidence. Moreover, a direct function of ABC1 transporters in interleukin 1 β secretion is rather unlikely as these proteins have been shown to be involved in lipid transport (Borst et al., 2000; Hamon et al., 2000; Schmitz and Langmann, 2001). In any case, our results suggest that FGF-2 and Gal-1 export do not involve the function of ABC transporters, provided that protein unfolding is a general prerequisite for ABC transporter-mediated protein translocation across membranes.

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