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Protein folding does not prevent the nonclassical export of FGF1 and S100A13

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Abstract

Newly synthesized proteins are usually exported through the endoplasmic reticulum (ER) and Golgi due to the presence in their primary sequence of a hydrophobic signal peptide that is recognized by the ER translocation system. However, some secreted proteins lack a signal peptide and are exported independently of ER-Golgi. Fibroblast growth factor (FGF)1 is included in this group of polypeptides, as well as S100A13 that is a small calcium-binding protein critical for FGF1 export. Classically secreted proteins are transported into ER in their unfolded states. To determine the role of protein tertiary structure in FGF1 export through the cell membrane, we produced the chimeras of FGF1 and S100A13 with dihydrofolate reductase (DHFR). The specific DHFR inhibitor, aminopterin, prevents its unfolding. We found that aminopterin did not inhibit the release of FGF1:DHFR and S100A13:DHFR. Thus, FGF1 and S100A13 can be exported in folded conformation.

Keywords

FGF1; S100A13; nonclassical release; protein folding; dihydrofolate reductase; aminopterin

INTRODUCTION

Most secreted proteins have in their primary structure a short (7–25 aminoacids) N-terminal hydrophobic signal peptide (SP) that allows the ribosome-protein complex to be recognized by a signal recognition particle (SRP) in the cytosol [1]. Through the GTP-dependent interaction between the SRP and SRP receptor at the endoplasmic reticulum (ER) membrane, the ribosome docks onto the translocon channel, and the nascent protein is released into the ER compartment [1]. Nascent proteins co-translationally enter ER in unfolded conformation, and subsequent folding and covalent modifications occur in the ER and Golgi before the protein

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is released through the exocytotic fusion of a Golgi-derived secretory vesicle with the cell membrane. Mitochondrial proteins synthesized in the cytosol post-translationally translocate through mitochondrial membranes also in unfolded conformation, using TOM and TIM translocases [2].

The ER-Golgi-dependent secretion pathway is usually referred to as classical or conventional. Secreted proteins that lack a SP generally cannot enter the ER, and their release is not attenuated by Brefeldin A that interferes with ER-Golgi organization. Their secretion pathways are defined as nonclassical or unconventional [3]. Unconventionally secreted proteins include the fibroblast growth factor (FGF)1 [4] and FGF2 [5–7], interleukin (IL)1α [8] and IL1β [9,10], macrophage migration inhibitory factor [11], morphogen epimorphin [12], HSP70 [13,14] and HSP90 [15], S100 proteins [16,17], sphingosine kinase 1, [18,19], thioredoxin [20,21], chromatin-associated proteins HMBG1 [22] and Engrailed 2 [23,24], secretory transglutaminase [25], annexins I [26] and II [27,28], galectins [29–31], and some secreted viral [32–34] and protozoan [35] polypeptides. While some of these proteins exhibit constitutive release, others are secreted upon specific stimulation [36]. Secretion of different SP-less proteins varies in its sensitivity to chemical inhibitors [3,36], suggesting that unconventional secretion is not a uniform process.

FGF1 release is induced by cellular stress conditions such as heat shock [4], hypoxia [37], serum starvation [38], and oxidized LDL treatment [39]. FGF1 is released from the cells as a copper-dependent multiprotein complex that includes a small calcium-binding protein S100A13 [40,41]. Unlike FGF1, this accessory protein can be constitutively released from the cells. However, when coexpressed with FGF1, its export becomes stress-dependent [36].

The use of a dominant negative mutant of S100A13 demonstrated the importance of this protein for FGF1 export [41]. Particularly interesting, S100A13 participates also in the stress-dependent nonclassical export of IL1α [42] and specifically associates with FGF1 at stress in glial cells [43]. Our confocal microscopy study demonstrated that under stress, FGF1 and S100A13 colocalize at the plasma membrane [44]. How these proteins are translocated through the cell membrane, and what is their conformational status during this process remain to be determined.

Studies based on the production of dihydrofolate reductase (DHFR)-containing chimeras are conducted to clarify the role of unfolding in protein translocation across the biological membranes [45]. This method is based on the use of a specific DHFR inhibitor aminopterin (AP) that stabilizes the tertiary structure of the enzyme or its chimeras, and prevents their transport across biological membranes if this transport requires protein unfolding [45]. Using this method, Nickel et al. demonstrated that FGF2 does not require total unfolding for its unconventional export [7]. However, FGF1 and FGF2 are released through distinct pathways [3,46]. Indeed, unlike FGF1, FGF2 is exported constitutively, and its export is sensitive to the inhibitors of Na^+/K^+ ATPase [6]. Therefore, we used the DHFR chimera approach to determine whether protein unfolding is a prerequisite of the release of FGF1 and the critical component of FGF1 release complex, S100A13. We found that complete unfolding is not required for the unconventional export of FGF1 and S100A13.

MATERIALS AND METHODS

Production of FGF1:DHFR and 6Myc:S100A13:DHFR chimeras

The original murine DHFR construct was generously gifted by Walter Nickel, University of Heidelberg, Germany. The DHFR cDNA was excised by digestion with BsrGI and PvuII. A BsrGI restriction site was introduced by PCR mutagenesis (Quick Mutagenesis Kit, Stratagene) at the C-termini of FGF1:HA and 6Myc:S100A13 genes in their respective expression

plasmids. FGF1α [47] form was used as in all the previous FGF1 export experiments of our laboratory [36,48]. After mutagenesis, FGF1:HA TOPO construct (generous gift of Andrew Baird, Human BioMolecular Research Institute, San Diego, CA) and 6Myc:S100A13 pcDNA 3.1 Hygro [41] were digested with BsrGI and Pvu II, and the DHFR fragment was ligated in frame with FGF1:HA and 6Myc:S100A13. The DHFR chimeras constructs were sequenced using the DNA Sequencing Kit (Applied Biosystems) according to manufacturer's instruction.

DHFR chimeras expression in NIH 3T3 cells and their stabilization by aminopterin

NIH 3T3 cells were grown on 10 cm Petri dishes in DMEM containing 10% Bovine Calf Serum (Hyclone) and 1% antimycotic antibiotic solution (GIBCO) to 60% confluency, and then transfected by using the JetPei transfectant reagent (QBiogene) with 1 μg DNA per dish of FGF1 (the above mentioned FGF1:HA construct was used), FGF1:DHFR, 6Myc:S100A13 or 6Myc:S100A13:DHFR. Twenty-four hours after transfection, the cells were collected and lysed in 100 mM Hepes-KOH pH 7.4, 2 mM $CaCl_2$, 0.2% Triton X100. The cell lysates (CL) were resolved by SDS-PAGE, blotted with rabbit anti-FGF1 antibody [4] for FGF1 and FGF1:DHFR or rabbit anti-Myc antibodies (Covance) for 6Myc-S100A13 and 6Myc:S100A13:DHFR, and developed by using the ECL kit (Roche). In trypsin protection experiments, CL were treated with 200 μg/ml trypsin in the presence or absence of 50 μM AP. The CL were resolved by SDS-PAGE and blotted with rabbit anti-DHFR antibody (Sigma).

Study of DHFR chimeras secretion

NIH 3T3 cells were transiently transfected with FGF1:DHFR or with 6Myc:S100A13:DHFR, as described above. Twenty four hours later, each 10 cm dish was split into four 15 cm dishes. Two days later, the cells were incubated for 4 hours in complete culture medium with either 1mM AP or with DMSO (solvent control). Then, heat shock was performed in DMEM containing 5U/ml heparin and either 1 mM AP or DMSO. Conditioned media from cultures incubated for 2 hours at 37°C and 42°C were filtered and treated with 0.1% dithiotreitol at 37° C for 2 hours. FGF1:DHFR was purified from the conditioned media by using heparin chromatography as described [4], whereas 6Myc:S100A13:DHFR was purified by immunoprecipitation [41] with monoclonal anti-Myc antibody (Covance). CL were prepared as controls of protein expression. The CL and conditioned media were analyzed by SDS-PAGE and immunoblotted as described above.

Immunofluorescence control of aminopterin efficiency (prevention of DHFR transmembrane translocation)

NIH 3T3 cells grown on fibronectin-coated glass coverslips were transfected with a reporter construct coding for the chimera comprised of the mitochondrial transport sequence (MTS), GFP and DHFR (MTS:GFP:DHFR) that was generously gifted by Walter Nickel, University of Heidelberg, Germany. Eight hours from transfection, the cells were incubated for four hours in complete culture medium with either 1 mM AP or DMSO. Then, the cells were fixed in 10% formalin for 10 min. Confocal microscopy (LTCS-SP microscope, Leica) was used to observe MTS:GFP:DHFR intracellular distribution in the presence or absence of AP.

RESULTS AND DISCUSSION

The exact mechanism of transmembrane translocation of FGF1 is unknown. To understand this mechanism, it is important to clarify the role of tertiary conformation of FGF1 release pathway components in this process. Indeed, translocation of proteins into the ER and mitochondria is based upon their unfolded conformation [1,2]. To elucidate whether the nonclassical export of FGF1 requires unfolding of FGF1 and S100A13, we applied the method of DHFR tagging of the released proteins [45]. FGF1 and S100A13, a member of both the FGF1 and IL1α release complexes, were C-terminally tagged with DHFR (Figure 1A), which

is an enzyme locked in folded conformation by its specific inhibitor AP [45]. FGF1:DHFR and 6Myc:S100A13:DHFR chimeras were transfected to NIH 3T3 cells and their expression was verified by using, respectively anti-FGF1, and anti-Myc antibodies (Figure 1B). The AP-induced conformational change of the DHFR moiety of chimeras was assessed based on its sensitivity to trypsin-induced proteolysis. Lysates of cells transfected with chimeras were treated with trypsin in the presence and absence of AP, resolved by SDS-PAGE, and immunoblotted using anti-DHFR antibodies. Trypsin treatment resulted in the proteolysis of the chimeras, whereas some free DHFR moiety was still detectable (Figure 1C). AP strongly increased the amount of the trypsin-resistant DHFR moiety.

The immunoblotting of the conditioned medium with anti-DHFR antibodies demonstrated that 1 mM AP treatment did not attenuate the release of FGF1:DHFR from NIH 3T3 cells following heat shock (Figure 2A). AP also did not interfere with the spontaneous export of S100A13:DHFR chimera (Figure 2B). Conversely, 1 mM AP applied to NIH3T3 cells blocked the mitochondrial localization of the GFP:DHFR chimera tagged to the MTS (Figure 2C). Thus, protein folding does not interfere with the nonclassical release of S100A13 and FGF1.

The results of DHFR chimera and experiments indicate that FGF1 and S100A13 export does not require total protein unfolding. How membrane translocation of unfolded proteins proceed without impairing cell viability remains to be determined. Other nonclassically released proteins, such as IL1β and HMBG1 [10,22] overcome this problem by translocation into lysosome-like vesicles, which further fuse with the cell membrane and release their contents into the extracellular compartment. However, confocal microscopy demonstrated diffuse but not punctate (vesicular) distribution of FGF1 and S100A13 in the cytoplasm [44]. Moreover, recombinant FGF2 translocates through the isolated plasma membrane [7], and we recently obtained similar results with FGF1(unpublished data). Based on the specific interaction of members of the FGF1 release complex with acidic pL [49], we hypothesized [36] that these proteins could be co-exported with acidic phospholipids during their stress-induced translocation from the inner to the outer leaflet of the cell membrane. Recently, Hirai et al. [12] demonstrated that the nonclassical export of epimorphin is associated with the transmembrane translocation of phosphatidylserine. Our results permit further consideration of the problem of nonclassical release of FGF1 and S100A13 from the angle of tertiary protein structure. Significantly, several nonclassically released proteins such as FGF1, FGF2, IL1α and IL1β [3] present a β–barrel in their structure that is also found in many integral membrane proteins [50]. Several members of S100 protein family are nonclassically released, and they all exhibit two characteristic EF-hand domains in their structure [51]. The results of the present study prompt us to further explore the role of specific features of the three-dimensional organization of FGF1 and S100A13 (β-barrel and EF-hands, respectively) in the nonclassical export of these proteins and their interaction with the phospholipid bilayers.

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Abbreviations

AP

Aminopterin

DHFR

Dihydrofolate Reductase

ER

Endoplasmic Reticulum

FGF

Fibroblast Growth Factor

IL1

Interleukin 1

MTS

Mitochondrial Transport Sequence

SP

Signal Peptide

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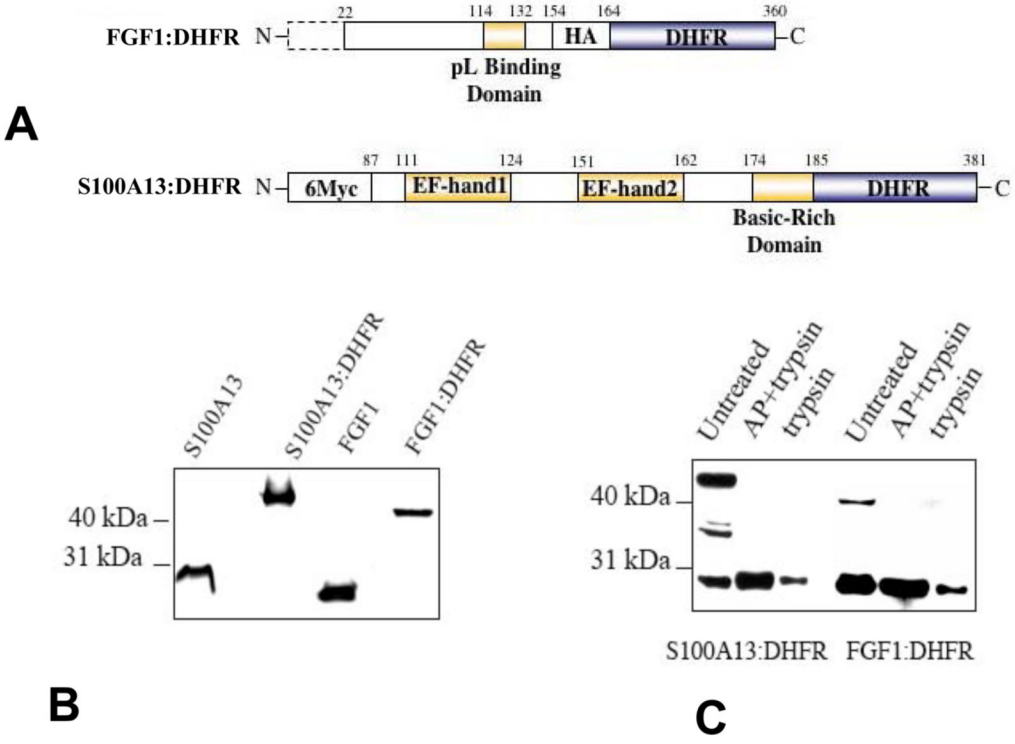

Figure 1. FGF1:DHFR and S100A13:DHFR chimeras

(A) Structures of FGF1:DHFR and 6Myc:S100:DHFR chimeras. Dotted line (residues 1–21 of FGF1) indicates the residues present in the FGF1β form and absent from the FGF1α form used in our experiments. **(B)** Expression of FGF1 (FGF1:HA construct was used), FGF1:DHFR, 6Myc:S100A13 and 6Myc:S100A13:DHFR in NIH 3T3 cells, 24 hours after transfection. Cell lysates (CL) were resolved by SDS-PAGE and blotted with anti-FGF1 or anti-Myc antibodies. **(C)**. CL prepared from NIH 3T3 cultures transiently transfected with FGF1:DHFR and 6Myc:S100A13:DHFR were treated with 200 μg/ml trypsin in the presence or absence of 50 μM AP. Untreated CL were used as controls. CL were resolved by SDS-PAGE and immunoblotted with anti-DHFR antibodies.

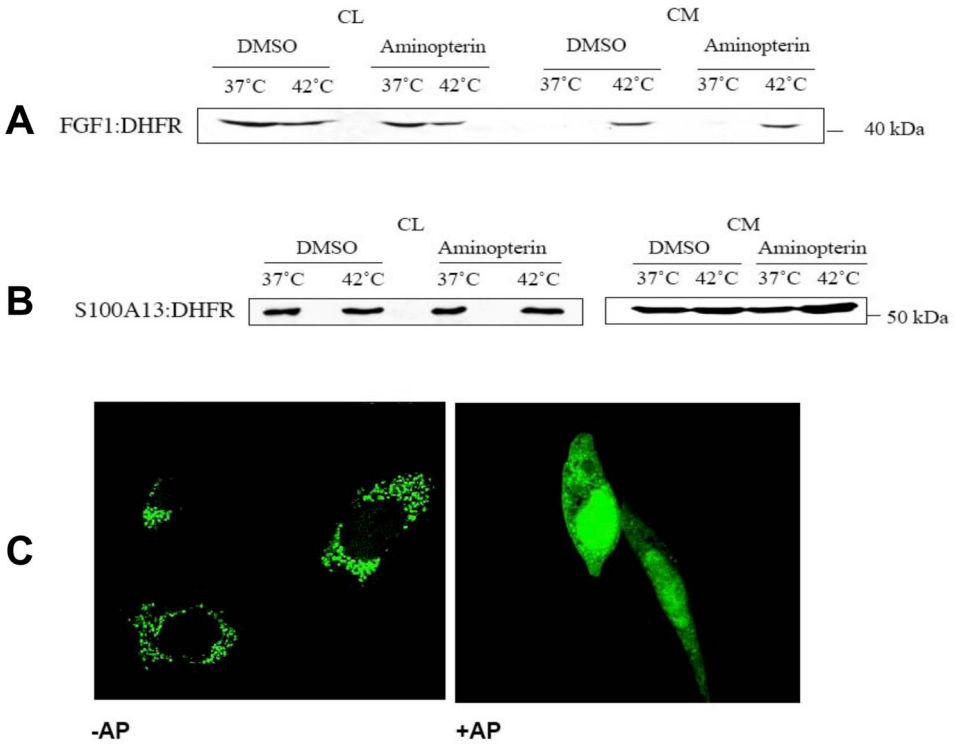

Figure 2. AP does not prevent the secretion of FGF1:DHFR and 6Myc:S100A13:DHFR

NIH 3T3 cells were transiently transfected with FGF1:DHFR **(A)** or 6Myc:S100A13:DHFR (**B**). Transfected cells were pre-incubated for 4 hours with 1 mM AP or with DMSO (solvent control). Then, cells were incubated for 2 hours at 37°C or 42°C, and CL and conditioned media (CM) were collected. FGF1:DHFR and 6Myc:S100A13:DHFR were isolated from CM, resolved by SDS-PAGE and immunoblotted with anti-DHFR antibodies. **(C)**. AP in the concentration of 1 mM abolishes mitochondrial translocation of MTS:GFP:DHFR reporter. NIH 3T3 cells were transfected with MTS:GFP:DHFR. AP was added to the cells 8 h after transfection. Cells were incubated for additional 4 h with or without AP and then fixed. Confocal microscopy (objective X63) demonstrated that incubation with AP results in diffuse cytosolic and nuclear distribution of MTS:GFP:DHFR instead of punctate mitochondrial.