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Release of FGF1 and p40 synaptotagmin 1 correlates with their membrane destabilizing ability ☆

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The article is dedicated to the memory of Tom Maciag, friend, scientist, and mentor.

Abstract

Fibroblast growth factor (FGF)1 is released from cells as a constituent of a complex that contains the small calcium binding protein S100A13, and the p40 kDa form of synaptotagmin (Syt)1, through an ER–Golgi-independent stress-induced pathway. FGF1 and the other components of its secretory complex are signal peptide-less proteins. We examined their capability to interact with lipid bilayers by studying protein-induced carboxyfluorescein release from liposomes of different phospholipid (pL) compositions. FGF1, p40 Syt1, and S100A13 induced destabilization of liposomes composed of acidic but not of zwitterionic pL. We produced mutants of FGF1 and p40 Syt1, in which specific basic amino acid residues in the regions that bind acidic pL were substituted. The ability of these mutants to induce liposomes destabilization was strongly attenuated, and they exhibited drastically diminished spontaneous and stress-induced release. Apparently, the non-classical release of FGF1 and p40 Syt1 involves destabilization of membranes containing acidic pL.
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Fibroblast growth factor (FGF)1 regulates embryonic development of vertebrates [1] and plays important roles in angiogenesis, inflammation, wound healing, and as a neurotrophic factor [2,3]. Similar to another ubiquitous and biologically important prototype member of the FGF family, FGF2, FGF1 belongs to a large group of proteins that lack a conventional signal sequence and gain access to the extracellular compartment independently of the endoplasmic reticulum (ER)–Golgi apparatus [4–13]. Indeed, FGF1 release is insensitive to Brefeldin A [14], which blocks ER-to-Golgi vesicular transport [15], and FGF1 does not appear to be present in the cytoplasmic vesicles [16]. Thus, FGF1 export through exocytotic fusion of secretory vesicles with the cell membrane is unlikely. FGF1 is secreted from cells upon stress stimulation such as heat shock [14], hypoxia [17], serum starvation [18], and treatment with oxidized LDL [19]. The availability of free intracellular copper ions is necessary for FGF1 release, and *in vitro* data suggest the formation of a copper- and stress-dependent multiprotein export complex [20], which contains the calcium-binding proteins, S100A13 and p40

Syt1 [21,22]. p40 Syt1 is a non-transmembrane isoform of the integral component of secretory vesicles, synaptotagmin (Syt)1, that is involved in the fusion of exocytotic vesicles with the plasma membrane [23]. Our laboratory demonstrated that p40 Syt1 is produced by alternative initiation of translation of p65 Syt1 mRNA [24].

Both p40 Syt1 and S100A13 display a constitutive and stress-induced release from transfected NIH 3T3 cells. Their constitutive release is blocked when they are cotransfected into the cells along with FGF1; however upon stress, they are released in a complex with FGF1 [21,22].

Although significant progress has been achieved in the study of non-classical protein release, the final step that allows these polypeptides to translocate across the cell membrane remains elusive. Confocal immunofluorescence microscopy studies demonstrated that in response to stress, cytosolic FGF1, S100A13, and p40 Syt1 traffic to the inner surface of the plasma membrane of the cells which co-express them [16]. It was suggested that the assembly of the FGF1 release complex may occur in this locale through the interaction of the individual polypeptide components with membrane phospholipids (pL). Indeed, it is known that: (i) FGF1 is able to bind acidic pL in a solid phase pL assay [25]; (ii) Syt1 can bind phosphatidylserine (pS) through its C2A domain and phosphorylated forms of phosphatidylinositol (pI) through its C2B domain [26]; and (iii) some members of the S100 family, such as S100A6 and S100A10, bind pL and they are important regulators of the functions of pL-binding proteins [27,28]. However, the ability of S100A13 to interact with pL has not been evaluated.

FGF1 disrupts acidic pL-containing liposome integrity [29], is able to deform lipid bilayers [30], and it exhibits molten globule (MG) character at temperatures above 30 °C [31,32], at acidic pH and in the presence of acidic pL [29]. MG is a partially unfolded conformation that bestows more hydrophobic features on the protein, and therefore increases the capability to interact with lipids [33]. Given the pL binding capability and pL-induced MG state of FGF1 [25,29], we investigated whether specific membrane pL play a role in FGF1 non-classical release. To this end, we studied the interaction of FGF1, S100A13, and p40 Syt1 with liposomes of various pL compositions. Our results demonstrated that the three members of the FGF1 release complex altered the integrity of membranes composed of acidic pL. Based on these data, we found that the mutations of specific basic amino acid residues in the C2B domain of p40 Syt1 and in the C-terminal part of FGF1 attenuated their ability to destabilize liposomes and blocked or drastically decreased their release from the cells.

Materials and methods

Materials. Dioleoylphosphatidylinositol (pI), dioleoylphosphatidylglycerol (pG), dioleoylphosphatidylserine (pS), and dioleoylphosphatidylcholine (pC) were purchased from Avanti Polar Lipid (Alabaster, AL). The fluorescent dye, 5,6-carboxyfluorescein (CF), was purchased from Molecular Probes, Inc. (Eugene, OR).

Plasmids and recombinant proteins. The plasmids for eukaryotic expression of FGF1 (pXZ38) and p40 Syt1 (p40-Syt1:Myc in pMEX hygro) were prepared as previously described [14,21,22]. The K326,327,331Q mutant of p40 Syt1 was produced by mutagenesis from the p40 Syt1:Myc pMEX Hygro and GST-p40 Syt1-pGEX-KG original plasmids [22,34] for eukaryotic and prokayotic expression, respectively. FGF1 K126,127A and FGF1 K114,115A mutants were produced by mutagenesis of the FGF1:HA pCR3.1 construct (gift of Andrew Baird, Human BioMolecular Research Institute, San Diego, CA) for eukaryotic expression, and the FGF1 pET3C construct [35] for prokaryotic expression. Recombinant FGF1, S100A13, and p40 Syt1 were produced and purified by HPLC as previously described [21,34,35].

Liposome preparation and fluorescence measurement. CF-loaded unilammelar liposomes were prepared as previously described [36]. The fluorescence of liposomes resuspended in 10 mM HEPES, 150 mM NaCl (pH 7.0) was monitored for 10 min by a Fluorolog-3 spectrofluorimeter (Jobin Yvon Horiba, Edison, NJ) at an excitation wavelength 470 nm and an emission wavelength 520 nm at 50 °C, as previously described [29,36]. Different concentrations of FGF1, S100A13, and p40 Syt1 recombinant proteins were added to the cuvette at the second minute of the experiment.

Cell culture, heat shock, conditioned media processing, and immunoblot analysis. NIH 3T3 cells were grown to 70% confluence in Dulbecco's modified Eagle's medium (DMEM; HyClone) supplemented with 10% (v/v) bovine calf serum (BCS, HyClone) and 1X antibiotic/antimycotic mixture (Gibco), on human fibronectin-coated (10 μg/cm^2) 10 cm dishes (Corning). For transient transfections, p40 Syt1 WT, p40 K326,327,331Q Syt1, FGF1:HA WT, FGF1:HA K114,115A or FGF1:HA K126,127A DNA were used in combination with the JetPEI transfectant reagent according to the manufacturer's instructions (Qbiogene Inc.).

Transiently transfected NIH 3T3 cells grown to 70–80% confluency were washed with DMEM containing 5 units/ml heparin (Sigma), and heat shock was performed as previously described [14] in DMEM containing 5 units/ml heparin for 110 min at 42 °C; control cultures were incubated at 37 °C in the same medium. Released p40 Syt1 was isolated using heparin Sepharose chromatography as described [22]. Released FGF1:HA was immunoprecipitated using anti-HA monoclonal antibodies (Covance) as described [21]. FGF1 and p40 Syt1 were resolved by SDS PAGE and detected by immunoblotting as described [14,22].

Confocal microscopy analysis. NIH 3T3 cells were plated on fibronectin-coated glass coverslips in 6-well plates, grown to 70% confluence, and then transiently transfected with 1 μg per well of either p40 Syt1:Myc, p40 Syt1:Myc K326,327,331Q, FGF1:HA, FGF1:HA K114,115A, or FGF1:HA K126,127A DNA, as described above. After 24 h, the culture medium was substituted with DMEM containing 5 units/ml heparin, and heat shock was performed as described above. The cells were fixed with 4% formaldehyde and immunostained as previously described [16] using a monoclonal anti-Myc antibody (Oncogene) or a monoclonal anti-HA antibody (Covance) followed by a fluorescein-conjugated anti-mouse IgG antibody and Hoechst 34580 (both from Molecular Probes). The cells were examined using a 100× objective of a LTCS-SP confocal system (Leica) at a 237 μM confocal pinhole.

Results

Proteins of the FGF1 release complex induce liposome destabilization that is dependent on liposome composition and protein concentration

Liposomes loaded with fluorescent molecules represent an established model for studying the membrane destabilizing activity of proteins [37]. FGF1 destabilized mixed pG/pC liposomes [29]. To investigate the interaction of FGF1 with various plasma membrane pL, we

compared the destabilizing effect of FGF1 on liposomes consisting of several acidic (pS, pI, pG) and a zwitterionic (pC) pL. We also evaluated whether other components of the FGF1 release pathway, i.e., S100A13 and p40 Syt1, induce liposome destabilization (Fig. 1A–C). The recombinant forms of FGF1, S100A13, and p40 Syt1 were added individually to the pL liposome suspension in the cuvette, and the CF release was detected fluorimetrically. The release of CF from liposomes results in a dequenching of fluorescence due to a sharp decrease of CF concentration [29]. The final protein concentrations in the cuvette were 1 μM, 0.5 μM, 0.25 μM, 0.125 μM. The pL concentration was 2 μM. We used α-chymotrypsin (Sigma) at the maximal concentration employed for studied proteins as a standard negative control because it is unable to induce liposome leakage [29], and 0.1% Triton X-100 served as a positive control for complete liposome destabilization (maximal CF release).

We found that FGF1 induced destabilization of pI, pG, and pS liposomes, but not the pC liposomes. The dependence of the ability of FGF1 to induce liposome destabilization upon their pL composition may be represented by the following relationship: pI > pG > pS > pC (Fig. 1A).

S100A13 exhibited destabilizing effects on liposomes with a similar relationship as FGF1: pG > pI > pS > pC (Fig. 1B). Conversely, p40 Syt1 was not able to induce any CF release from pC, pS, and pG liposomes, but very efficiently destabilized pI liposomes (Fig. 1C). It is important to stress that none of the proteins were able to induce CF release from zwitterionic (pC) liposomes (Fig. 1A–C). The extent of CF release was proportional to protein concentration. Thus, S100A13 at 1 μM concentration induced CF release from pG liposomes almost equivalent to complete liposome destabilization induced by 0.1% Triton X-100 (Fig. 1B).

We concluded that the proteins of the FGF1 release complex were able to induce destabilization of liposomes composed of acidic pL, but not zwitterionic pL, in a protein concentration-dependent manner. In addition, for p40 Syt1, this ability exhibited selectivity towards a specific acidic pL (pI).

p40 Syt1 K326,327,331Q mutant is not released by NIH 3T3 cells

The ability of the three members of the FGF1 release complex to destabilize acidic pL membranes prompted us to use mutagenesis to investigate whether this property is related to non-classical release. To that end, we first chose Syt1, whose interaction with membranes is extensively studied [26]. A loss of pI binding capability was described for a closely related protein Syt2 after substitution of three closely located lysine residues in the C2B domain [38]. Corresponding lysine residues of Syt1 bind PIP$_2$, a pI derivate that is present at the plasma membrane [39]. We mutated
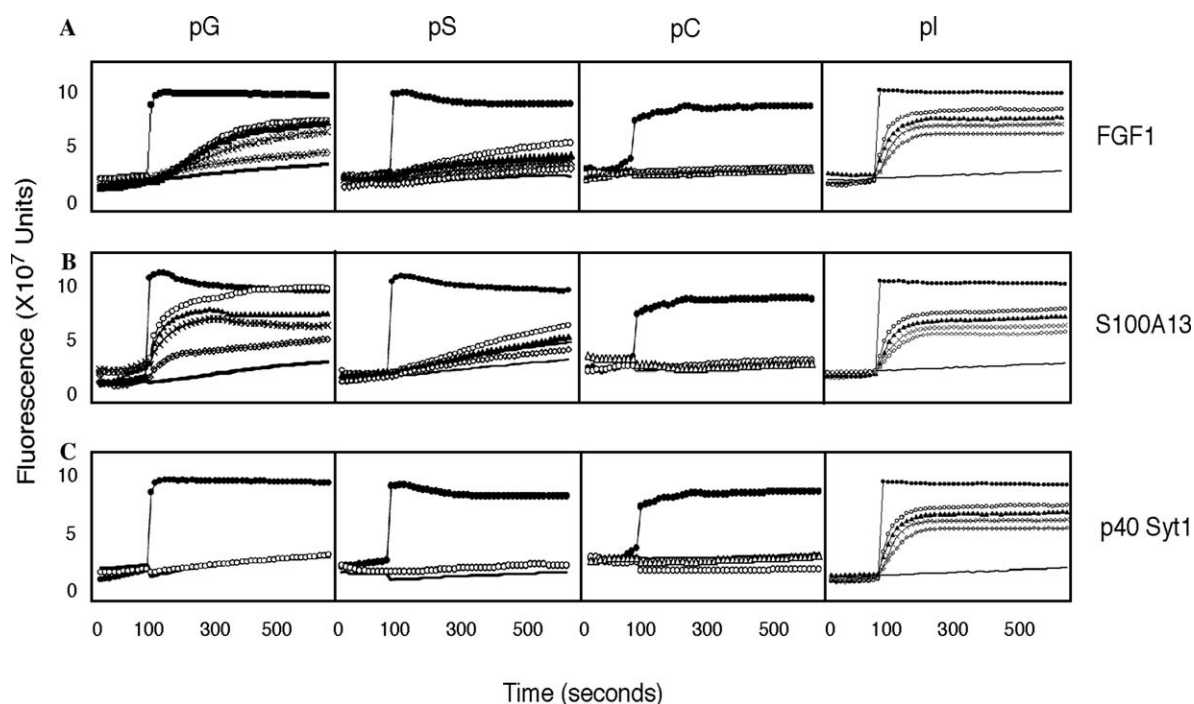

Fig. 1. The release of carboxyfluorescein (CF) from pL liposomes by FGF1 (A), S100A13 (B), and p40 Syt1 (C). Liposomes were prepared using pS, pG, pI, or pC in the presence of CF. The release of CF after the addition of 1 mM (○-○), 500 nM (Δ-Δ), 250 nM (×-×), 125 nM (◇-◇) of recombinant FGF1, S100A13, and p40 Syt1 was continuously monitored using a fluorescence spectrophotometer (excitation: 470 nm; emission: 520 nm). The data are reported as a function of time in seconds. One millimolar α-chymotrypsin (—) or 0.1% Triton X-100 (●-●) were used as, respectively, negative and positive controls of membrane destabiliization. The pL concentration in the cuvette was 2 μM.

these lysine residues (K326, 327 and 331) in the C2B domain of p40 Syt1, in order to evaluate the release of the resultant mutant during stress and at normal conditions. Heat shock experiments performed on NIH 3T3 cells transiently transfected with either the mutant or the WT form of p40 Syt1 demonstrated that, unlike the WT form, the p40 Syt1 K326,327,331Q mutant was not released
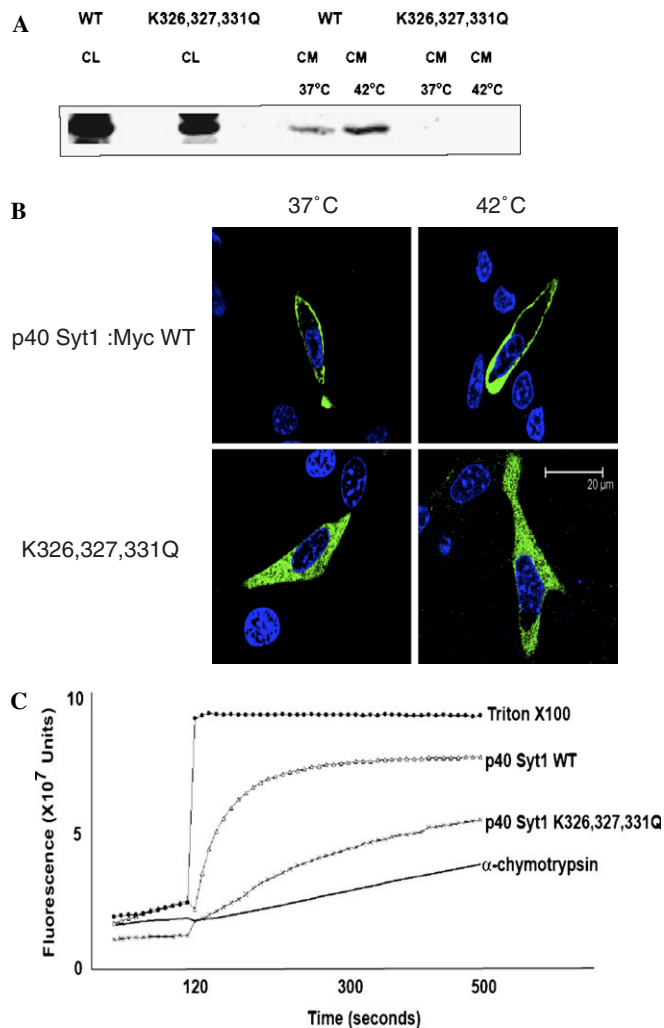

Fig. 2. (A) The release of p40 Syt1 WT and p40 Syt1 K326,327,331Q from NIH 3T3 cells. NIH 3T3 cells transfected with p40 Syt1 WT or p40 Syt1 K326,327,321Q were incubated for 2 h at 37 °C or 42 °C. p40 Syt1 from conditioned medium was adsorbed to heparin–Sepharose and analyzed by immunoblotting as described [14]. CL – cell lysate; CM – conditioned medium. (B) Confocal fluorescence microscopy analysis of p40 Syt1:Myc WT and p40 Syt1:Myc K326,327,331Q cellular localization at normal and stress conditions. NIH 3T3 cells transfected with either p40 Syt1:Myc WT or p40 Syt1:Myc K326,327,331Q were incubated for 2 h at 37 °C or 42 °C. The cells were fixed with formaldehyde and immunofluorescently stained for Myc tag (green) as described in the "Materials and methods". The nuclei were stained with Hoechst 34580 (blue). The cells were studied using a confocal microscope with an X100 objective. Median horizontal confocal cell sections are presented. Bar – 20 μm. (C) Analysis of the CF release from pI liposomes induced by p40 Syt1 WT and p40 Syt1 K326,327,331Q. The experiments on CF release from pI liposomes induced by 500 nM p40 Syt1 WT or p40 Syt1 K326,327,331Q were performed as described in Fig. 1.

either at 37 °C or 42 °C (Fig. 2A). This suggests that the lysine residues at positions 326, 327, 331 are required for the non-classical release of p40 Syt1 from cells.

p40 Syt1 K326,327,331Q does not display enhanced perimembrane localization in transfected cell

Previously, we demonstrated that when the WT form of p40 Syt1 is co-expressed with FGF1, it migrates to the plasma membrane under stress conditions like other members of the FGF1 release complex [16]. Since p40 Syt1 K326,327,331Q is not released by NIH 3T3 cells, we evaluated its localization by confocal fluorescence microscopy during heat shock and at normal conditions in transiently transfected NIH 3T3 cells. Our study demonstrated that p40 Syt1 K326,327,331Q presented a diffuse cytosolic distribution pattern both at 37 °C and 42 °C (Fig. 2B), which was unlike the WT form that displayed an enhanced localization in the vicinity of the cell membrane under both conditions (Fig. 2B).

K326,327,331Q mutations in the p40 Syt1 C2B domain lead to a sharp decrease of pI liposome destabilization activity

The inability of p40 Syt1 K326,327,331Q to be released by NIH 3T3 cells and to exhibit preferential localization in the vicinity of the plasma membrane, prompted us to explore its liposome permeabilizing activity. The p40 Syt1 K326,327,331Q-induced CF release from pI liposomes was strongly reduced compared to WT p40 Syt1 (Fig. 2C). These data suggest that the lysine residues at positions 326, 327, and 331 play a crucial role in the ability of p40 Syt1 to bind the plasma membrane, destabilize it, and exit to the extracellular compartment.

FGF1 K114,115A, and FGF1 K126,127A exhibit strongly attenuated release from NIH 3T3 cells

Since the C-terminal region of FGF1 is responsible for interaction with acidic pL [25] and we demonstrated that the substitution of a lysine cluster in p40 Syt1 results in the loss of the non-classical release of this protein from NIH 3T3 cells, we investigated the role of lysine clusters in the C-terminal region of FGF1 in FGF1 release, and its ability to destabilize acidic pL membranes. For this purpose, we produced two FGF1 mutants, FGF1 K114,115A and FGF1 K126,127A, and we tested their ability to be released by transiently transfected NIH 3T3 cells after heat shock. These experiments demonstrated that FGF1 K114,115A exhibited a drastically reduced release at 42 °C as compared to the WT FGF1, while FGF1 K126,127 was not released at this temperature (Fig. 3A). These data suggest that the clusters of lysine residues in the FGF1 C-terminal domain are important for stress-induced release of this growth factor from cells.
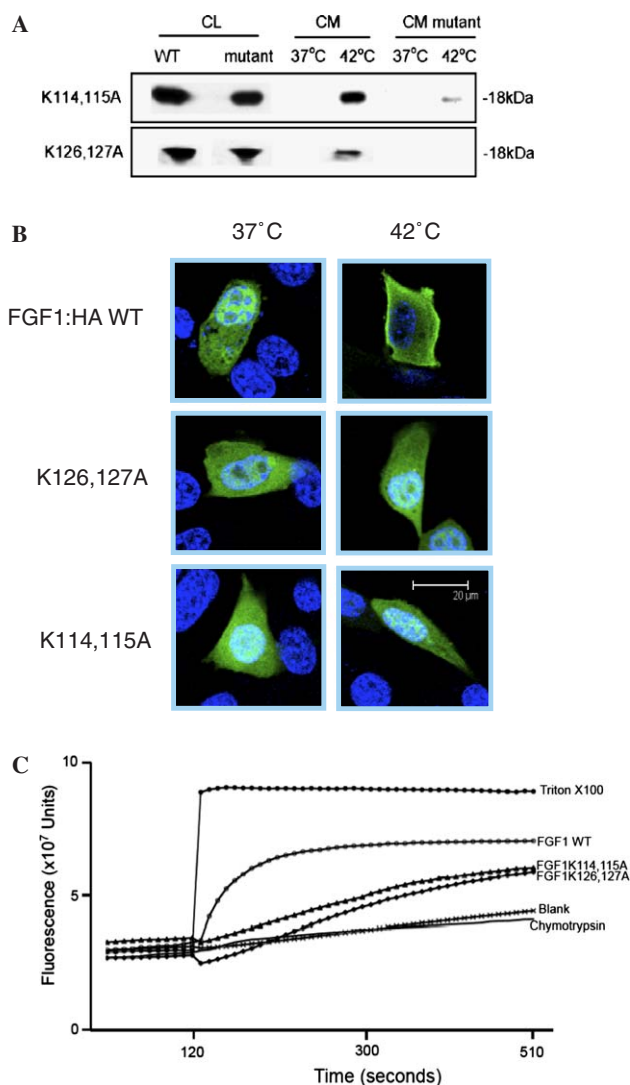
Fig. 3. (A) The release of FGF1 WT, FGF1 K114,115A, and FGF1 K126,127A from NIH 3T3 cells. NIH 3T3 cells transfected with FGF1:HA WT, FGF1:HA K114,115A or FGF1:HA K126,127A were incubated for 2 h at 37 °C or 42 °C. FGF1:HA from conditioned medium was immunoprecipitated and analyzed by immunoblotting as described in Materials and methods. CL – cell lysate; CM – conditioned medium. (B) Confocal fluorescence microscopy analysis of FGF1:HA WT, FGF1:HA K114,115A and FGF1:HA K126,127A cellular localization at normal and stress conditions. NIH 3T3 cells transfected with either FGF1:HA WT, FGF1:HA K114,115A, or FGF1:HA K126,127A were incubated for 2 h at 37 °C or 42 °C. Cells were fixed with formaldehyde and immunofluorescently stained for HA tag (green) as described in the Materials and Methods. Nuclei were stained with Hoechst 34580 (blue). The cells were studied using a confocal microscope with a X100 objective. Median horizontal confocal cell sections are presented. Bar – 20 μm. (C) Analysis of the CF release from pG liposomes induced by FGF1 WT, FGF1 K114,115A, or FGF1 K126,127A. The experiments on CF release from pG liposomes induced by 500 nM FGF1 WT, FGF1 K114,115A, or FGF1 K126,127A were performed as described in Fig. 1.

FGF1 K114,115A and FGF1 K126,127A do not display perimembrane redistribution at heat shock

We previously demonstrated that FGF1 and the components of its secretory pathway migrate toward the plasma membrane prior to release [16]. Since FGF1:HA K114,115A and FGF1:HA K126,127A, like p40 Syt1 K326,327,331Q, are not released from NIH 3T3 cells, we studied their intracellular localization under normal and stress conditions in order to define whether their impaired release was related to a defect in the association with the cell membrane. After transfecting NIH 3T3 cell cultures with either the mutants or the WT form of FGF1 and submitting them to heat shock, we performed immunofluorescence staining for the HA tag and studied the cells using confocal microscopy. We found (Fig. 3B) that contrary to FGF1 WT, neither FGF1:HA K114,115A nor FGF1:HA K126,127A exhibited enhanced perimembrane localization after heat shock.

K114,115A or K126,127A mutations in the C-terminal region of FGF1 lead to the decrease of its ability to destabilize liposomes

To determine whether the attenuated secretion of FGF1:HA K114,115A and FGF1:HA K126,127A correlates with the decreased ability to destabilize acidic pL liposomes, we produced recombinant forms of FGF1 K114,115A and FGF1 K126,127A mutants and tested their effects on CF-loaded acidic pL liposomes. Recombinant FGF1 K114,115A and FGF1 K126,127A (Fig. 3C) demonstrated a strong decrease of destabilizing activity towards pG liposomes as compared to destabilizing activity of the recombinant WT FGF1, suggesting that lysine clusters at the C-terminal domain of FGF1 are critical for both membrane destabilization and non-classical protein release.

Discussion

Despite the absence of a classical signal sequence in its structure, FGF1 exhibits stress-induced release [14]. However, the mechanism used by FGF1 to cross the cell membrane is not known. Previously it was demonstrated that FGF1 binds acidic pL [25], and is able to deform lipid bilayers [30]. Moreover, FGF1 destabilizes liposomes containing acidic pL and assumes a partially unfolded MG-like conformation in the presence of acidic pL [29]. In the MG-like state conformation, FGF1 retains most of its secondary structure but its tertiary structure is almost completely disrupted. The solvent-exposed non-polar surface(s) in the MG-like state probably facilitates the interaction of FGF1 with the lipid membrane. Subsequently, the bound FGF1 appears to disorganize the lipid membrane, due to acquired hydrophobic characteristics that may facilitate its solubility in membranes and perturb their integrity [29,33]. However, since FGF1 is released in association with S100A13 and p40 Syt1 [21,22], the question arised whether the two latter proteins could also display membrane destabilizing properties. Similar to FGF1 [25], Syt1 is able to associate with acidic pL, particularly pS and pI [26]. In addition, some S100 family members bind

pL or pL-binding proteins [27,28]. These previous observations prompted us to undertake a comparative study of the interaction of FGF1, p40 Syt1, and S100A13 with artificial membranes composed of different pL, to determine whether individual members of the FGF1 release complex exhibit preferential membrane destabilizing abilities dependent upon specific pL membrane composition. We chose pC, a zwitterionic pL, and three acidic pL - pI, pG, and pS. All of these pL are present in the plasma membrane [40–42], and acidic pL are preferentially localized in the plasma membrane inner leaflet [40]. We observed that the three proteins destabilized lipid membranes composed of acidic pL but not of pC, which is a zwitterionic pL. While FGF1 and S100A13 exhibited destabilizing activity towards pG, pS, and pI membranes, p40 Syt1 induced selective destabilization of only the pI liposomes.

Since we previously demonstrated that when the components of the FGF1 release complex are co-expressed, they localize at the plasma membrane in response to stress [16], the observation that FGF1 release complex members destabilize acidic pL liposomes suggests a critical role for plasma membrane pL in the assembly and export of the FGF1 release complex [12]. Particularly, the preference exhibited by p40 Syt1 in permeabilizing pI liposomes indicates the existence of sites of specific pL compositions that recruit different constituents of the FGF1 release complex at the inner leaflet of the plasma membrane. Therefore, it is likely that specific pL function as anchors for individual proteins, promoting the formation of the multiprotein FGF1 release complex at the inner leaflet of the cell membrane. Additionally, we should consider the interaction between acidic pL and the components of the FGF1 secretion pathway in terms of reciprocal structural changes: acidic pL might be responsible for inducing tertiary structure modifications that allow the proteins to become more hydrophobic [29]; on the other hand, the proteins might be responsible of membrane deformation [30]. Thus, both of these mechanisms might ultimately contribute to protein release. This might be the case of p40 Syt1, which binds pS and pI, respectively, through the C2A and C2B domains [26], but exhibits destabilizing activity only towards pI liposomes. It is also important to mention that the ability of some pL, i.e., pS, to flip from the inner to the outer face of the plasma membrane in response to stress [43,44] might be involved in the protein translocation process.

The calcium-binding protein, S1001A13, which is released from cells in association with FGF1 [21], and similar to FGF1 exhibits destabilizing activity upon acidic pL liposomes (Fig. 2), is a member of the S100 proteins family. S100 proteins regulate the functions of their protein partners through induced conformational changes. In particular, S100A10 regulates the process of insertion of annexin II into the plasma membrane [45]. Similar to S100A10, S100A13 combined with acidic pL might promote FGF1 and p40 Syt1 conformational changes, and translocation of the FGF1 release complex across the plasma membrane.

The establishment of membrane destabilizing properties of the FGF1 release complex components prompted us to use mutational analysis to identify the membrane destabilizing domains of p40 Syt1 and FGF1. To this purpose, we used available information regarding the acidic pL-binding domains of synaptotagmins [38,39] and FGF1 [25]. We found that mutation of lysines 326, 327, and 331 to glutamines resulted in the inability of p40 Syt1 to be released from NIH 3T3 cells both at 37 °C and at 42 °C, as well as a strong decrease of pI liposome destabilization. Additionally, whereas p40 Syt1 WT exhibited perimembrane localization, p40 Syt1 K326,327,331Q was distributed diffusely in the cytoplasm of transfected cells. Similarly, substitution of lysine residues in the C-terminal domain of FGF1 abolished its capability to be released from NIH 3T3 cells, to localize at the plasma membrane under stress conditions, and to perturb acidic pL bilayer integrity. Thus, clusters of lysine residues (i) are responsible for the interaction of p40 Syt1 and FGF1 with the plasma membrane, (ii) are required for their non-classical release, (iii) are at least partially responsible for the destabilization of acidic pL membranes.

In conclusion, this work demonstrated that the members of non-classical FGF1 release complex exhibited the ability to destabilize artificial membranes composed of acidic pL, which are preferentially localized in the inner leaflet of the cell membrane [46, Micheva, 2001 #3641]. Moreover, the membrane destabilizing ability of p40 Syt1 and FGF1 correlated with their non-classical release from NIH 3T3 cells. Additionally, we previously reported that interleukin 1α, a protein exported through a non-classical release pathway similar to FGF1, also efficiently permeabilizes pG liposomes [36]. Further identification of the membrane destabilizing domains of S100A13 and structural characterization of the interaction of the FGF1 release complex components with lipid membranes is important for the understanding of the mechanism of non-classical protein release, which may be applied to the rational design of therapeutic compounds that interfere with this process.

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