Phosphatidylinositol 4,5-Bisphosphate (PI(4,5)P$_2$)-dependent Oligomerization of Fibroblast Growth Factor 2 (FGF2) Triggers the Formation of a Lipidic Membrane Pore Implicated in Unconventional Secretion$^{*,5}$

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Background: PI(4,5)P$_2$- and tyrosine phosphorylation-dependent unconventional secretion of FGF2 is mediated by direct translocation across the plasma membrane.

Results: PI(4,5)P$_2$-mediated membrane recruitment causes oligomerization of tyrosine-phosphorylated FGF2 that, in turn, triggers the formation of a lipidic membrane pore.

Conclusion: Membrane-inserted FGF2 oligomers represent intermediates of membrane translocation during unconventional secretion.

Significance: Mechanistic insight into a novel self-sustained mechanism of protein translocation across membranes is provided.

Fibroblast growth factor 2 (FGF2) is a critical mitogen with a central role in specific steps of tumor-induced angiogenesis. It is known to be secreted by unconventional means bypassing the endoplasmic reticulum/Golgi-dependent secretory pathway. However, the mechanism of FGF2 membrane translocation into the extracellular space has remained elusive. Here, we show that phosphatidylinositol 4,5-bisphosphate-dependent membrane recruitment causes FGF2 to oligomerize, which in turn triggers the formation of a lipidic membrane pore with a putative toroidal structure. This process is strongly up-regulated by tyrosine phosphorylation of FGF2. Our findings explain key requirements of FGF2 secretion from living cells and suggest a novel self-sustained mechanism of protein translocation across membranes with a lipidic membrane pore being a transient translocation intermediate.

Proteins secreted by unconventional means do not contain N-terminal signal peptides and therefore do not have access to the lumen of the endoplasmic reticulum/Golgi-dependent secretory pathway (1–3). Classical examples are FGF2 and interleukin 1β (II1β) (4) defining the two major types of unconventional secretory pathways. FGF2 is exported from cells by direct translocation across plasma membranes (type I unconventional secretion) (5, 6). By contrast, secretion of II1β was reported to involve intracellular vesicular transport intermediates (type II unconventional secretion) (4, 7–11). Besides FGF2, additional extracellular factors have been classified as type I unconventional secretory proteins such as FGF1, HIV Tat, and annexin A2 (12–14). In addition to II1β, further examples of type II unconventional secretion are acyl-CoA-binding protein (AcbA/Acb1) and tissue transglutaminase (3, 15–20).

FGF2 was shown to translocate across plasma membranes (5, 6), a process that is initiated by its recruitment at the inner leaflet mediated by the phosphoinositide PI(4,5)P$_2$ (21, 22). At the outer leaflet, membrane-proximal heparan sulfate proteoglycans are involved in a late step of FGF2 secretion resulting in its exposure on cell surfaces (23, 24). These sequential interactions are essential, as experimental conditions that prevent the ability of FGF2 to interact with either PI(4,5)P$_2$ or heparan sulfates cause inhibition of FGF2 secretion (22, 24, 25). Both of these interactions require FGF2 to be folded properly (26), and FGF2 also remains folded during membrane translocation (26, 27). Thus, we previously proposed that FGF2 membrane translocation is not mediated by a classical protein-conducting channel but rather involves some kind of an alternative mechanism of membrane translocation (25). The dependence of this process on a folded conformation has further been interpreted as an intrinsic quality control mechanism of FGF2 secretion (26, 28).

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$^3$ The abbreviations used are: PI(4,5)P$_2$, phosphatidylinositol 4,5-bisphosphate; FCCS, fluorescence cross-correlation spectroscopy; Ni-NTA, nickel-nitrilotriacetic acid; GUV, giant unilamellar vesicle; CF, carboxyfluorescein; PE, phosphatidylethanolamine; PIP$_2$, phosphatidylinositol 4,5-bisphosphate.
Mechanism of FGF2 Membrane Translocation

Another component of the unconventional secretory machinery of FGF2 is Tec kinase, a protein that was found in a genome-wide RNAi screen designed to identify novel components of FGF2 secretion (29). Tec kinase was demonstrated to phosphorylate FGF2 at tyrosine 82, a modification required for efficient secretion of FGF2 (25, 29); however, the mechanism by which FGF2 physically traverses the plasma membrane and by which tyrosine phosphorylation affects FGF2 secretion remains unknown.

Using biochemical flotation experiments and scanning fluorescence cross-correlation spectroscopy, we demonstrate that FGF2 oligomerizes in a PI(4,5)P$_2$-dependent manner at membrane surfaces. Based on membrane passage of small fluorescent tracer molecules and transbilayer diffusion of membrane lipids, we find that PI(4,5)P$_2$-dependent oligomerization of FGF2 drives the formation of a lipidic membrane pore (30). We further demonstrate that this process is vastly stimulated by tyrosine phosphorylation suggesting stabilization of a specific oligomeric conformation of FGF2 with a high potential to trigger the formation of lipidic membrane pores. Diacylglycerol, a membrane lipid with the reverse shape of PI(4,5)P$_2$, inhibited FGF2-dependent pore formation. This finding demonstrates that, in addition to FGF2 oligomerization and tyrosine phosphorylation, positive membrane curvature induced by PI(4,5)P$_2$ is required for this process. Consistent with the observation of transbilayer diffusion of membrane lipids, this finding suggests the formation of a lipidic membrane pore with a putative toroidal architecture. Thus, the reconstitution experiments of this study suggest a novel self-sustained mechanism of membrane translocation with a lipidic membrane pore being a transient translocation intermediate. These findings explain the behavior of FGF2 in living cells where secretion depends on PI(4,5)P$_2$-mediated membrane recruitment (21, 22, 28), tyrosine phosphorylation (25, 29), and proper folding of FGF2 (26, 27).

EXPERIMENTAL PROCEDURES

Protein Purification—Recombinant FGF2 fusion proteins were expressed in *Escherichia coli* using the expression vectors pQE30 (Qiagen) (FGF2-WT and FGF2-PIP$_2$-Mut (K128Q, R129Q, K134Q)) and pET-15b (FGF2-Y82pCMF). In case of FGF2-Y82pCMF, codon 82 (tyrosine) was replaced by an amber stop codon followed by transformation of *E. coli* BL21 Star (DE3) carrying the plasmid pEVOL-pCMF (31). Protein expression was performed in 2 × YT medium containing 1 mm $p$-carboxymethylphenylalanine (custom synthesis by ENAMINE Ltd.) for 16 h at 25 °C as described (31). All proteins were Histagged at the N terminus and were affinity-purified using standard procedures.

Preparation of Liposomes—All lipids were purchased from Avanti Polar Lipids. Phosphatidylcholine, phosphatidylinositol, and phosphatidylethanolamine (PE) were from bovine liver; phosphatidyserine and phosphatidylinositol-4,5-bisphosphate (PI(4,5)P$_2$) were derived from porcine brain; cholesterol was from ovine wool, and sphingomyelin was from poultry eggs. Diacylglycerol (1-palmitoyl-2-oleoyl-sn-glycerol), lissamine rhodamine B-labeled PE (16:0), and 1,2-dioleoyl-sn-glycerol-3-[[N-(5-amino-1-carboxypentyl) iminodiacetic acid] succinyl]; nickel salt (18:1) were synthetic products.

Chloroform-dissolved lipid mixtures were first dried under a gentle nitrogen stream and further dried under vacuum for 1.5 h to yield a homogeneous lipid film. Lipids were resuspended in buffer A (150 mm KCl, 25 mm HEPE, pH 7.4, 10% (w/v) sucrose) at 45 °C to form liposomes with a final lipid concentration of 4 mm (biochemical interaction experiments and transbilayer diffusion experiments) or 8 mm (fluorescence depquenching experiments). Liposomes were subjected to 10 freeze/thaw cycles and 21 size extrusion steps (mini-extruder; Avanti Polar Lipids). Liposome preparations were analyzed by dynamic light scattering, indicating a range 200–400 nm in diameter.

Biochemical Analysis of Protein Binding to Liposomes—Lipo- somes with a plasma membrane-like lipid composition containing either 2 mol % PI(4,5)P$_2$ or 2 mol % of a Ni-NTA lipid were generated as described earlier (22, 32). Membranes were blocked with 3% (w/v) fatty acid-free BSA for 1 h at 25 °C and washed with buffer B (25 mm HEPE, 150 mm KCl, pH 7.4). Following incubation for 30 min at 25 °C with FGF2-WT, FGF2-Y82pCMF, or FGF2-PIP$_2$-Mut (K128Q, R129Q, and K134Q), liposomes were reisolated by flotation using Nycodenz density gradients (40:30:0% (w/v) in binding buffer) as described in Ref. 33. About 2.25% of input, bound and unbound material, was analyzed by SDS-PAGE under reducing conditions and Western blotting using anti-FGF2 antibodies.

Quantification of PI(4,5)P$_2$-dependent FGF2 Oligomerization on Membrane Surfaces of Giant Unilamellar Vesicles Using Scanning Fluorescence Cross-correlation Spectroscopy (FCCS)—Giant unilamellar vesicles with a plasma membrane-like lipid composition containing either PI(4,5)P$_2$ or a Ni-NTA lipid were generated as described below. His-tagged forms of FGF2-WT and FGF2-Y82pCMF were labeled at cysteines with Atto488 maleimide or Atto655 maleimide dyes as described by the manufacturer (Atto TEC). Unbound dye was removed with Econo-Pac 10DG desalting columns (Bio-Rad). The degree of labeling was determined by mass spectrometry based on relative peak intensities and found to be 90% for FGF2-Y82pCMF for both Atto488 and Atto655, respectively. For FGF2-WT, the labeling efficiency was 80% for Atto488 and 70% for Atto655 (supplemental Fig. S1).

Two-focus scanning FCCS measurements were conducted at 22 °C on an LSM710 confocal fluorescence microscope using a C-Apochromat 40 × 1.2 water immersion objective (Zeiss, Jena, Germany). Argon ion (488 nm) and HeNe (633 nm) lasers were used for sample excitation. Photon detection was done with the avalanche photodiodes of the ConFocor 3 module, and the photon arrival times were recorded in the photon mode of the hardware correlator Flex 02–01D/C. Measurements were done as described previously (34). Briefly, the detection volume was repeatedly scanned perpendicularly through the equator of a GUV in a frame mode with Nx2 pixels to scan the two parallel lines. Data analysis was performed with software written in MATLAB (34). The photon stream was binned in 2-μs bins and arranged as a matrix, with every row corresponding to one line scan. Movements of the membrane were corrected, and autocorrelation and spectral as well as spatial cross-correlation curves were determined. With the structural parameters and the distance between the two scanned lines being known, dif-
fusion coefficients were calculated under the experimental conditions indicated. Irregular curves resulting from instabilities and identified by distortions of the curves and a systematic change in the intensity trace were removed. Cross-correlation was corrected for cross-talk (5%) and for the degree of labeling as described previously (34).

**Analysis of Protein-dependent Membrane Permeabilizing Activity**—Liposomes with a plasma membrane-like lipid composition containing 2 mol % Pl(4,5)P₂ were prepared in buffer A (with a lowered KCl concentration of 100 mM) supplemented with 100 μM of the membrane-impermeant fluorophore 5(6)-carboxyfluorescein (CF; Sigma). To remove extraluminal CF, liposomes were diluted in buffer B and collected by centrifugation at 15,000 × g for 10 min at 20 °C followed by size exclusion chromatography using a PD10 column (GE Healthcare). Importantly, this column was operated in buffer C (150 mM KCl, 25 mM HEPES, pH 7.4, 10% (w/v) sucrose, 2% (w/v) glucose) that was titrated with glucose to reach iso-osmolality (840 mosmol/kg). Following incubation with proteins (1 μM) as indicated, fluorescence dequenching was measured using a Gemini XS (Molecular Devices) fluorescence plate reader. At the end of each experiment, Triton X-100 (0.2% (w/v) final concentration) was added to measure the maximum of dequenching that was used to normalize data. Fluorescence dequenching measured in the presence of buffer was considered background and was subtracted from all other measurements.

**Analysis of Protein Membrane Activity Using GUVs and Confocal Microscopy**—GUVs were prepared at 45 °C in sucrose (300 mosmol/kg) based on the electro-swelling method using platinum electrodes (10 Hz, 1.5 V for 40 min, 2 Hz, 1.5 V for 20 min (34)). A plasma membrane-like lipid composition consisting of 30 mol % cholesterol, 15 mol % sphingomyelin, 35 mol % phosphatidylcholine, 10 mol % PE, 5 mol % phosphatidylserine, 5 mol % phosphatidylinositol, and 0.05 mol % rhodamine B-labeled PE was supplemented with either Pl(4,5)P₂ or a Ni-NTA lipid (2 mol % at the expense of phosphatidylinositol) as indicated. GUVs were incubated with different fluorescent tracer molecules as indicated, Alexa488 (~1 kDa), dextran-Alexa488 (~10 kDa), and Alexa488-labeled cytochrome c (~12 kDa) in buffer B (305 mosmol/kg). Proteins were added to GUVs at a final concentration of 50 nM as indicated.

Confocal images were recorded at room temperature in multitrack or single track mode on a Zeiss LSM710 confocal fluorescence microscope using a C-Apochromat 40 × 1.2 water immersion objective. To detect the Alexa 488 dye, samples were excited with an argon ion laser (488 nm), and the light beam was split by a spectral beam guide to 493–536 nm. For rhodamine-PE, samples were excited with a He-Ne laser at 561 nm, and the light beam was split to 581–681 nm. Images were recorded in 8-bit grayscale, pseudo-colored in green (channel 1) and red (channel 2) followed by processing with ImageJ (rsweb.nih.gov). Luminal fluorescence of individual GUVs (~300 for each experimental condition) was measured and normalized to the fluorescence intensity of the surrounding buffer.

**Analysis of Transbilayer Diffusion of Pyrene-labeled Sphingomyelin**—Pyrene-labeled sphingomyelin was solubilized in ethanol (250 μl) and added to preformed liposomes with a diameter of about 100 nm at 5 mol % of total membrane lipids. Samples were incubated for 15 min at 37 °C to generate asymmetrically labeled liposomes. Fluorescence measurements were performed with a Jasco FP-6500 spectrofluorometer using quartz cuvettes. Measurements were conducted with an excitation wavelength of 344 nm. Emission wavelengths were 398 nm for monomers, and 474 nm for excimers. Incorporation into the outer membrane leaflet of pyrene-labeled sphingomyelin was determined by the increase of the monomer to excimer ratio at 398 and 474 nm, respectively, as a function of time.

The rate of transbilayer lipid diffusion was measured using the method described by Müller et al. (35). All experiments were performed at 37 °C under continuous stirring with a final lipid concentration of 20 μM for LUVs. Fluorescence intensities of excimers at 474 nm (Iₐ) and monomers at 398 nm (Iₐ) were determined from spectra taken at various time points after the addition of the various forms of FGF2 indicated. Iₐ/Iₐ ratios at the beginning of the experiment were set to 1. An Iₐ/Iₐ ratio of less than 1 demonstrates diffusion of pyrene-labeled sphingomyelin from the outer to the inner leaflet with Iₐ/Iₐ = 0.5 indicating complete equilibration between the two leaflets.

**RESULTS**

Pl(4,5)P₂-dependent Oligomerization of FGF2 on Membrane Surfaces—To explore potential mechanisms of FGF2 membrane translocation, we used three different His-tagged variants of FGF2, the wild-type form (FGF2-WT), phosphomimetic FGF2 containing the unnatural amino acid p-carboxyphenylalanine at position 82 (FGF2-Y82pCMF (36, 37)), and an FGF2 variant in which basic residues of the Pl(4,5)P₂ binding pocket were replaced by glutamines resulting in impaired Pl(4,5)P₂ binding (FGF2-PIP₂-Mut (22)). Two types of liposomes were generated that contained a plasma membrane-like lipid composition supplemented with either Pl(4,5)P₂ or a Ni-NTA lipid used to recruit His-tagged proteins.

Protein binding to liposomes was assessed by flotation gradients combined with an SDS-PAGE/Western blot analysis. As shown in Fig. 1, all three proteins bound efficiently to liposomes containing the Ni-NTA lipid (lanes 4, 9, and 14, respectively). By contrast, whereas FGF2-WT and FGF2-Y82pCMF bound efficiently to liposomes containing Pl(4,5)P₂ (Fig. 1, lanes 2 and 7, respectively), only trace amounts of FGF2-PIP₂-Mut were found associated with these membranes (lane 12).

Following binding of FGF2-WT and FGF2-Y82pCMF to Pl(4,5)P₂-containing liposomes, formation of SDS-resistant oligomers was observed (Fig. 1, lanes 2 and 7). Based on migration behavior, the largest detectable FGF2 oligomer represented a hexamer. By contrast, when FGF2-WT and FGF2-Y82pCMF were recruited via the Ni-NTA lipid, larger forms of FGF2 oligomers (tetramers, pentamers, or hexamers) could not be observed (Fig. 1, lanes 4 and 9). Similarly, only trace amounts of pentameric or hexameric oligomers were observed when FGF2-PIP₂-Mut was recruited to membranes using the Ni-NTA lipid anchor (Fig. 1, lane 14).

To analyze Pl(4,5)P₂-dependent oligomerization of FGF2 under native conditions, we conducted scanning FCCS to quantify protein-protein interactions within membranes (34, 38–40). Briefly, fluorescence correlation spectroscopy analyzes the temporal fluorescence fluctuations due to the diffu-
sion of individual molecules in and out of the focal volume of the microscope. The signal is then auto-correlated to calculate the so-called auto-correlation curves (green and orange curves in Fig. 2, A–D), which provide quantitative information about the concentration of fluorophores and their diffusion coefficient. The two-color version of the technique can be used to quantify complex formation. The dynamic codiffusion of the proteins of interest can be detected by cross-correlating their corresponding fluorescence fluctuations. The extent of complex formation is calculated from the amplitude of the cross-correlation curve (blue in Fig. 2, A–D) with respect to the auto-correlation curves.

Both FGF2-WT and FGF2-Y82pCMF were labeled with either Atto488 or Atto655 dyes resulting in four independent preparations of fluorescently labeled proteins. Labeling efficiencies were determined by mass spectrometry (supplemental Fig. S1). Mixtures of Atto488- and Atto655-labeled FGF2-WT or Atto488- and Atto655-labeled FGF2-Y82pCMF were added to GUVs with a plasma membrane-like lipid composition containing either PI(4,5)P₂ or a Ni-NTA lipid. Protein amounts were titrated to achieve similar binding efficiencies for all four experimental conditions shown in Fig. 2.

Based on a positive amplitude of the cross-correlation curves (Fig. 2, A and B, blue curves), both FGF2-WT and FGF2-Y82pCMF were found to self-associate when recruited via PI(4,5)P₂. By contrast, cross-correlation could not be observed when FGF2-WT and FGF2-Y82pCMF were recruited through the Ni-NTA lipid (Fig. 2, C and D, blue curves) demonstrating a lack of complex formation under these experimental conditions. The extent of complex formation under the different experimental conditions was calculated from the auto- and cross-correlation curves and is shown in Fig. 2E. These calculations revealed an average of about 60% of both FGF2-WT and FGF2-Y82pCMF found in complexes when recruited via PI(4,5)P₂. By contrast, only small amounts of FGF2-WT and FGF2-Y82pCMF were found in complexes when recruited via the Ni-NTA lipid. From this FCCS analysis, we also obtained the diffusion constants of FGF2 in the membrane for all four experimental conditions (Fig. 2F). As expected, diffusion constants were significantly smaller under conditions that promote self-association of FGF2.

The combined findings shown in Figs. 1 and 2 demonstrate that FGF2 oligomerizes at membrane surfaces in a PI(4,5)P₂-dependent manner. Phosphorylation of tyrosine 82 does not appear to affect the size of FGF2 oligomers as indicated by the results with the phosphomimetic variant form of FGF2, FGF2-Y82pCMF.

**PI(4,5)P₂ and Tyrosine Phosphorylation-dependent Membrane Activity of FGF2 Oligomers**—Based on the observations shown in Figs. 1 and 2, we hypothesized that PI(4,5)P₂-induced self-association of FGF2 might result in membrane insertion of FGF2 oligomers. To address this possibility, we analyzed whether this process causes local alterations of membrane integrity (from here on referred to as “membrane activity”). Liposomes were incubated with various forms of FGF2 in the presence of a small fluorescent tracer molecule (41). This assay is based on luminal inclusion of CF into liposomes at a concentration that causes self-quenching. Protein-dependent formation of membrane pores can be measured by the release of CF monitoring fluorescence dequenching.

As shown in Fig. 3A (red squares), recruitment of FGF2-Y82pCMF to PI(4,5)P₂-containing liposomes caused fast and
efficient release of about 45% of total CF at 90 min of incubation. By contrast, binding of FGF2-WT to PI(4,5)P2-containing liposomes resulted in a slow and modest activity with about 5% CF release (Fig. 3A, blue spheres). Although this activity of FGF2-WT was low compared with FGF2-Y82pCMF, it was still significantly higher compared with FGF2-PIP-Mut (Fig. 3A, violet triangles). Strikingly, even though all three proteins tested did bind with similar efficiency (Fig. 1), none of them, including FGF2-Y82pCMF, caused release of CF from plasma membrane-like liposomes in which PI(4,5)P2 had been replaced by the Ni-NTA lipid (Fig. 3B). A titration of FGF2-Y82pCMF (0.125–2 μM) in the presence of plasma membrane-like liposomes containing PI(4,5)P2 revealed that FGF2-Y82pCMF causes release of CF in a protein concentration-dependent manner (Fig. 3C).

**FIGURE 2.** Analysis of PI(4,5)P2-dependent FGF2 oligomerization at membrane surfaces using scanning fluorescence cross-correlation spectroscopy. GUVs were prepared with a plasma membrane-like lipid composition containing either PI(4,5)P2 or a Ni-NTA lipid as indicated. His-tagged variants of FGF2-WT and FGF2-Y82pCMF were labeled with Atto488 and Atto655 dyes resulting in four independent preparations of fluorescent proteins. Mixtures of proteins with the two different dyes were added to the two types of GUVs resulting in four experimental conditions. Based on the FCCS analysis, we obtained auto-correlation curves for the Atto488- (green) and Atto655 (orange)-labeled FGF2 proteins, whose amplitude is inversely proportional to the concentration of the corresponding molecules. The two-color analysis yields the cross-correlation curve (blue), which provides information about the concentration of molecules forming a complex. The results of the fitting of the auto- and cross-correlation curves can then be combined to calculate the percentage of molecules forming a complex in the sample as well as diffusion constants. For further details see “Experimental Procedures.” A–D, representative examples of auto- (green and orange) and cross-correlation curves (blue) derived from two-focus scanning FCCS measurements of individual GUVs under the conditions indicated are shown. Solid lines correspond to fitted curves and dashed lines to raw data. E, percentage of FGF2 molecules found in complexes following correction for channel cross-talk and degree of labeling. Standard deviations are shown. To assess statistical significance, an unpaired two-tailed t test was performed using GraphPad Prism 5.0c (ns = not significant; *, p value ≤0.05; **, p value ≤0.01; ***, p value ≤0.001). F, diffusion coefficient of FGF2 molecules in individual GUVs. Standard deviations are shown. To assess statistical significance an unpaired two-tailed t test was performed using GraphPad Prism 5.0c (ns = not significant; *, p value ≤0.05; **, p value ≤0.01; ***, p value ≤0.001).

**FIGURE 3.** FGF2 membrane activity depends on PI(4,5)P2-mediated membrane recruitment and tyrosine phosphorylation. Carboxyfluorescein was sequestered in liposomes containing a plasma membrane-like lipid composition supplemented with either PI(4,5)P2 (A and C) or a Ni-NTA lipid (B). Liposomes were incubated with FGF2-WT, FGF2-Y82pCMF, or FGF2-PIP-Mut as indicated. Membrane activity was measured by fluorescence dequenching as described under “Experimental Procedures.” The results shown are representative of four independent preparations of liposomes.
Mechanism of FGF2 Membrane Translocation

These findings establish a direct correlation between FGF2 membrane activity and FGF2 oligomerization. Furthermore, phosphorylation of tyrosine 82 appears to cause substantial changes in the biophysical properties of FGF2 resulting in an oligomeric structure with a high potential for the formation of membrane pores.

Analysis of PI(4,5)P$_2$ and Tyrosine Phosphorylation-dependent Membrane Activity of FGF2 Oligomers Employing Giant Unilamellar Vesicles and Confocal Microscopy—To directly visualize the membrane activity of FGF2-Y82pCMF, we employed GUVs combined with confocal microscopy (42). Three types of GUVs with a plasma membrane-like lipid composition either lacking PI(4,5)P$_2$, containing PI(4,5)P$_2$ (plasma membrane + PI$_P$), or containing the Ni-NTA lipid (plasma membrane + nickel) were generated (Fig. 4). GUVs were supplemented with rhodamine-PE to visualize their membranes. FGF2-Y82pCMF-dependent penetration into the lumen of GUVs of a fluorescent tracer molecule (Alexa488) was analyzed using FGF2-WT and FGF2-PI$_P$-Mut as controls.

Following incubation of FGF2-Y82pCMF with GUVs containing PI(4,5)P$_2$, the fluorescent tracer gained access to the lumen of a substantial population of vesicles without affecting their overall integrity (Fig. 4A, panel e, white arrows). By contrast, following incubation of FGF2-Y82pCMF with GUVs lacking PI(4,5)P$_2$, luminal penetration of Alexa488 could not be observed (Fig. 4A, panel d). Similarly, following incubation of FGF2-Y82pCMF with GUVs containing the Ni-NTA lipid, the fluorescent tracer was excluded from the lumen of GUVs (Fig. 4A, panel f). To quantify these experiments, individual GUVs were classified based on measurements of luminal fluorescence versus the fluorescence of the surroundings (Fig. 4B). Following incubation of PI(4,5)P$_2$ containing GUVs with FGF2-Y82pCMF, a substantial fraction of about 40% of total GUVs was characterized by high luminal fluorescence (≥80%) compared with the surroundings), indicating that the fluorescent tracer had equilibrated between the lumen and the surroundings of the vesicles. This behavior is typical for an “all-or-none” mechanism of protein-dependent membrane activity (42).

Quantification of control experiments using GUVs either lacking PI(4,5)P$_2$ or containing the Ni-NTA lipid revealed that, following incubation with FGF2-Y82pCMF, only a minor population contained the fluorescent tracer (Fig. 4B).

Based on the distribution of the various classes of GUVs shown in Fig. 4B, we quantified membrane activity of the proteins indicated using a threshold of ≥50% of luminal fluorescence compared with the surroundings (Fig. 4C). In addition to PI(4,5)P$_2$ dependence as demonstrated in Fig. 4B, this analysis revealed that, in the presence of GUVs containing PI(4,5)P$_2$, FGF2-WT exerted only low levels of membrane activity that was not significantly different from FGF2-PI$_P$-Mut used to define background (Fig. 4C). By contrast, FGF2-Y82pCMF exhibited a membrane activity that was significantly higher compared with both FGF2-WT and FGF2-PI$_P$-Mut. As documented by video microscopy analyzing individual GUVs in real time (supplemental Video S1), FGF2-Y82pCMF-mediated pore formation concomitant with luminal penetration of the fluorescent tracer did not affect the overall integrity of GUVs.

To analyze whether oligomers of FGF2-Y82pCMF cause the formation of membrane pores with a defined size cutoff, we compared membrane passage of various fluorescent tracers with different sizes and shapes (Fig. 5). GUVs with a plasma membrane-like lipid composition containing PI(4,5)P$_2$ were incubated with FGF2-Y82pCMF in the presence of either Alexa488 (−1 kDa; Fig. 5A), an Alexa488-labeled dextran molecule (∼10 kDa; Fig. 5B), or an Alexa488-labeled small globular protein, cytochrome c (∼12 kDa; Fig. 5C). Following incubation for 90 min with FGF2-Y82pCMF, the percentage of GUVs with a luminal fluorescence of ≥50% of the fluorescence compared with their surroundings was determined. In the presence of the 1-kDa fluorescent tracer, 25–30% of GUVs were positive for luminal fluorescence (Fig. 5D). By contrast, under the same experimental conditions, neither the 10-kDa dextran tracer nor the cytochrome c tracer was able to penetrate the lumen of GUVs to a significant extent (Fig. 5D). These findings establish that PI(4,5)P$_2$-dependent formation of FGF2-Y82pCMF oligomers results in membrane pores with a defined size cutoff.

PI(4,5)P$_2$-dependent Oligomerization of FGF2-Y82pCMF Triggers the Formation of a Lipidic Membrane Pore—It has been speculated that FGF2 membrane translocation involves the formation of a lipidic membrane pore (21, 25) with a toroidal structure being one possibility (30). The structural hallmark of a toroidal pore is that the two lipid monolayers of the membrane become connected allowing for exchange of membrane lipids between the two leaflets. To directly test this hypothesis, we analyzed transbilayer diffusion of pyrene-labeled phospholipids (35, 43, 44). These derivatives of membrane lipids form excimers (dimers) in a concentration-dependent manner. When added to preformed liposomes, pyrene-labeled phospholipids insert into the outer leaflet as a mixture of excimers and monomers resulting in the formation of asymmetrically labeled membranes. Upon formation of a protein-induced toroidal pore, membrane lipids can redistribute between the two bilayers. This causes dilution of the pyrene-labeled phospholipids shifting the equilibrium from excimers to monomers. This change can be measured due to the different spectral properties of these species. When FGF2-Y82pCMF was added to liposomes with a plasma membrane-like lipid composition containing PI(4,5)P$_2$, rapid transbilayer diffusion of pyrene-labeled sphingomyelin was observed (Fig. 6A) in a protein concentration-dependent manner (Fig. 6B). By contrast, FGF2-WT displayed only moderate activity (Fig. 6A). As expected, the PI(4,5)P$_2$-binding mutant form of FGF2 (FGF2-PIP$_2$-Mut) did not have any activity (Fig. 6A). The activity of FGF2-Y82pCMF could be inhibited by diacylglycerol (Fig. 6C), a molecule that compromises PI(4,5)P$_2$-induced positive membrane curvature due to its reverse shape. Finally, transbilayer diffusion of pyrene-labeled sphingomyelin induced by FGF2-Y82pCMF was not observed when PI(4,5)P$_2$ was replaced by a Ni-NTA lipid to prevent oligomerization of FGF2-Y82pCMF (Fig. 6C).

Our combined findings indicate that PI(4,5)P$_2$-dependent oligomerization of FGF2-Y82pCMF leads to the formation of lipidic membrane pores with a defined size cutoff and can permit diffusion of membrane lipids between the two monolayers. This suggests the formation of a toroidal pore structure as illustrated in Fig. 7.
FIGURE 4. Membrane activity of FGF2-Y82pCMF measured by confocal microscopy using giant unilamellar vesicles. A, confocal images of GUVs with a plasma membrane-like lipid composition either lacking PI(4,5)P₂ (panels a and d), containing PI(4,5)P₂ (panels b and e), or containing the Ni-NTA lipid (panels c and f). All three types of GUVs contained rhodamine B-labeled PE to visualize their membranes (red). Following formation of GUVs, a small fluorescent tracer molecule (Alexa 488, green) was added to monitor a potential membrane activity of added proteins measured by penetration of the lumen of GUVs. GUVs were incubated either in the presence of buffer (panels a–c) or FGF2-Y82pCMF (panels d–f). Following 90 min of incubation, confocal images were recorded (bar, 10 μm). B, quantitative analysis of the experiments shown in A (FGF2-Y82pCMF, panels d–f). GUVs were classified according to their luminal fluorescence compared with the surrounding fluorescence. Cyan bars represent GUVs lacking PI(4,5)P₂. Red bars represent GUVs containing PI(4,5)P₂. Blue bars represent GUVs containing the Ni-NTA lipid. For all conditions, data were derived from at least four independent experiments each of which involved the analysis of ≥300 GUVs per experimental condition. C, quantitative comparison and statistical analysis of membrane activity exerted by FGF2-Y82pCMF using FGF2-WT and FGF2-PIP-Mut as controls. Membrane activity is given as the percentage of GUVs with a luminal fluorescence of ≥50% compared with the surrounding fluorescence. For each experimental condition, the buffer control was subtracted. Standard deviations are shown. To assess statistical significance, an unpaired two-tailed t test was performed using GraphPad Prism 5.0c (ns, not significant; *, p value ≤0.05; **, p value ≤0.01).
**DISCUSSION**

FGF2 secretion from living cells occurs by direct protein translocation across plasma membranes (5, 24). This process depends on PI(4,5)P₂-mediated recruitment at the inner leaflet of the plasma membrane (22), tyrosine phosphorylation (29), and maintenance of folding during FGF2 membrane translocation (26, 27). Using a reconstituted system using model membranes and recombinant proteins, we now provide insight into a biochemical mechanism explaining these observations from cells. When recruited to membrane surfaces with a plasma membrane-like lipid composition containing PI(4,5)P₂, FGF2 assembled into oligomers with a hexamer being the largest detectable structure. By contrast, oligomerization failed when FGF2 was recruited via a His tag and a Ni-NTA lipid used as an

![Image](image-url)

**FIGURE 5.** FGF2-Y82pCMF generates pores in GUVs containing PI(4,5)P₂ with a defined size cutoff. GUVs with a plasma membrane-like lipid composition containing PI(4,5)P₂ were incubated in the presence of FGF2-Y82pCMF and either Alexa488 (1 kDa), Alexa488-labeled dextran (10 kDa), or Alexa488 labeled cytochrome c (12 kDa). Confocal imaging was conducted as described in the legend to Fig. 4. Luminal penetration of the fluorescent tracers indicated was quantified as described in the legend to Fig. 4 and under “Experimental Procedures” using a threshold of ≥50% of luminal fluorescence compared with the surroundings (D).

**FIGURE 6.** PI(4,5)P₂-dependent oligomerization of FGF2-Y82pCMF causes the formation of a toroidal membrane pore as measured by transbilayer diffusion of a membrane lipid. Transbilayer redistribution of pyrene-labeled sphingomyelin in large unilamellar liposomes was analyzed in the presence of either FGF2-Y82pCMF, the wild-type form of FGF2, a PI(4,5)P₂-binding mutant of FGF2, or buffer as indicated. Liposomes contained a plasma membrane (PM)-like lipid composition supplemented with either 2 mol% PI(4,5)P₂ or 2 mol% of the Ni-NTA lipid. Where indicated, liposomes were further supplemented with 5 mol% diacylglycerol (DAG). Following liposome formation, pyrene-labeled sphingomyelin was inserted into the outer leaflet resulting in asymmetrically labeled membranes. Addition of protein was carried out at t = 0 min. The time-dependent decrease of the $I_{IM}$/$I_E$ ratio indicates diffusion of pyrene-labeled sphingomyelin from the outer to the inner leaflet of liposomes. For further details, see “Experimental Procedures.” A quantitative comparison of transbilayer diffusion of pyrene-labeled sphingomyelin in the presence of either 1 μM FGF2-Y82pCMF, 1 μM FGF2-WT, or 1 μM FGF2-PIP-Mut using liposomes with a plasma membrane-like lipid composition containing PI(4,5)P₂, B, protein concentration-dependent transbilayer diffusion of pyrene-labeled sphingomyelin caused by FGF2-Y82pCMF using liposomes with a plasma membrane-like lipid composition containing PI(4,5)P₂, C, dependence of transbilayer diffusion of pyrene-labeled sphingomyelin on PI(4,5)P₂-mediated recruitment of FGF2-Y82pCMF (1 μM) and inhibition by diacylglycerol.
artificial membrane anchor. Similar results were obtained under native conditions using scanning fluorescence cross-correlation spectroscopy to analyze FGF2 oligomerization within membranes under various experimental conditions. This suggests that PI(4,5)P₂-dependent membrane recruitment orients FGF2 molecules in a specific way, promoting oligomerization. PI(4,5)P₂-dependent oligomerization of FGF2 was correlated with the formation of membrane pores with a defined size cutoff as measured by membrane passage of small fluorescent tracer molecules. This pore forming activity of FGF2 oligomers was vastly increased in an FGF2 variant in which tyrosine 82 was replaced by the phosphomimetic amino acid p-carboxymethylphenylalanine. Using a transbilayer diffusion assay monitoring mobility of membrane lipids between the two leaflets of model membranes, we provide evidence that PI(4,5)P₂-dependent oligomerization of FGF2-Y82pCMF results in the formation of a lipidic membrane pore with a putative toroidal architecture. We propose that this structure represents a transient translocation intermediate and disassembles at the extracellular side resulting in heparan sulfate proteoglycan-dependent exposure of FGF2 on cell surfaces (Fig. 7). Thus, our findings suggest a novel self-sustained mechanism of protein translocation across membranes.

Based on the results of this study as well as previous findings, we propose a model for the overall mechanism of FGF2 secretion. FGF2 and Tec kinase are recruited at the inner leafet of plasma membranes mediated by the phosphoinositides PI(4,5)P₂ (22, 28) and phosphatidylinositol 3,4,5-trisphosphate (45), respectively. Recruitment by PI(4,5)P₂ causes FGF2 to oligomerize. Simultaneously, membrane recruitment activates Tec kinase (46) resulting in phosphorylation of FGF2 at tyrosine 82 (25, 29). This modification may stabilize a specific oligomeric conformation of FGF2 with a high potential for the formation of a lipidic membrane pore as a transient translocation intermediate (Fig. 7). At this stage, heparan sulfate proteoglycan-dependent translocation of FGF2 into the extracellular space can occur (23, 24) resulting in the disassembly of the membrane pore. Because FGF2 has about a hundredfold higher affinity to heparan sulfate proteoglycans compared with PI(4,5)P₂ (22) and binding of FGF2 to either PI(4,5)P₂ or heparan sulfates is mutually exclusive, we propose that heparan sulfate proteoglycans liberate FGF2 from the membrane-associated state resulting in its exposure on cell surfaces.

Based on the detection of membrane-associated FGF2 hexamers (Fig. 1) and the triangular crystal structure of FGF2 monomers (47–49), one possibility for a pore-forming FGF2 oligomer would be a hexameric ring with the headgroups of PI(4,5)P₂ surrounding the FGF2 subunits (Fig. 7). A ring-like structure of FGF2 oligomers would also minimize the energy required to trigger the formation of a toroidal membrane pore. In this context, cone-shaped PI(4,5)P₂ membrane lipids at the site of FGF2 recruitment are likely to play a critical role in stabilizing positive membrane curvature. This interpretation is supported by the fact that diacylglycerol inhibits the formation of membrane pores triggered by PI(4,5)P₂-dependent oligomerization of FGF2 (Fig. 6).

Lipidic membrane pores are also induced by Bax, a protein involved in apoptosis that can permeabilize the outer membrane of mitochondria (30, 43, 44). Bax pores are stable structures and serve the irreversible destruction of the outer mitochondrial membrane in the course of programmed cell death (50). By contrast, we suggest that membrane pores triggered by FGF2 are highly dynamic and short lived structures that represent intermediates of FGF2 membrane translocation. In this sense, we suggest that the formation of a transient membrane pore triggered by FGF2 does not necessarily have a substantial impact on the overall integrity of the plasma membrane. For example, in a cellular context, additional factors such as membrane proteins may serve as transbilayer diffusion barriers of membrane lipids and may also prevent membrane passage of small molecules such as calcium ions. Nevertheless, they appear to share structural similarities with lipidic pores triggered by Bax. A hallmark of these structures is a lipidic inner surface that is covered by the headgroups of membrane lipids (30). Because of this, a toroidal pore is considered to have structural flexibility. In case of FGF2, this is consistent with previous observations demonstrating that various unrelated domains such as GFP can be fused to FGF2 without a complete loss of membrane translocation activity (24, 26, 51).

In conclusion, this study provides a biochemical and biophysical explanation for observations in living cells where FGF2 secretion was shown to occur by direct translocation across the plasma membrane (5, 24) as well as to depend on PI(4,5)P₂-mediated membrane recruitment (22), tyrosine phosphorylation (29), and maintenance of a folded conformation of FGF2 (26, 27). They are likely to also be relevant for other type I
unconventional secretory proteins such as HIV-Tat and AnxA2 (25). These proteins have been shown to interact with PI(4,5)P₂, and in the case of HIV Tat, this interaction was shown to be essential for unconventional secretion (14, 52). Furthermore, AnxA2 was demonstrated to require tyrosine phosphorylation for translocation to cell surfaces (13), a hallmark of the unconventional secretory mechanism of FGF2 (25, 29).

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