An intrinsic quality-control mechanism ensures unconventional secretion of fibroblast growth factor 2 in a folded conformation

Lucía Cespón Torrado[1], Koen Temmerman[1], Hans-Michael Müller[1], Matthias P. Mayer[2], Claudia Seelenmeyer[1], Rafael Backhaus[1] and Walter Nickel[1,*]

[1]Heidelberg University Biochemistry Center, Im Neuenheimer Feld 328, 69120 Heidelberg, Germany
[2]Zentrum für Molekulare Biologie der Universität Heidelberg, Im Neuenheimer Feld 282, 69120 Heidelberg, Germany
*Author for correspondence (walter.nickel@bzh.uni-heidelberg.de)

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Summary

Fibroblast growth factor 2 (FGF2) is a proangiogenic mitogen that is secreted by an unconventional mechanism, which does not depend on a functional ER-Golgi system. FGF2 is first recruited to the inner leaflet of plasma membranes, in a process that is mediated by the phosphoinositide PtdIns(4,5)P_2. On the extracellular side, membrane-proximal FGF2-binding sites provided by heparan-sulfate proteoglycans are essential for trapping and accumulating FGF2 in the extracellular space. Here we demonstrate that FGF2 membrane translocation can occur in a folded conformation, i.e. unfolded molecules are not obligatory intermediates in FGF2 secretion. Furthermore, we find that initial sorting into its export pathway requires FGF2 to be folded, because the interaction with PtdIns(4,5)P_2 is lost upon unfolding of FGF2. Our combined findings suggest an intrinsic quality-control mechanism that ensures extracellular accumulation of FGF2 in a biologically active form.

Key words: Unconventional protein secretion, Non-classical export, Membrane translocation, Fibroblast growth factor 2, FGF2, Protein folding, Quality control

Introduction

The molecular mechanisms of unconventional secretory processes have been a long-standing mystery in molecular cell biology (Muesch et al., 1990; Cleves, 1997; Hughes, 1999; Nickel, 2003; Prudovsky et al., 2008). Examples have been reported in almost all eukaryotes (Nickel, 2003; Prudovsky et al., 2008) including simple organisms such as *Dictyostelium* (Sesaki et al., 1997; Anjard and Loomis, 2005; Anjard and Loomis, 2006; Kinseth et al., 2007). Among unconventional secretory proteins are a number of important cytokines that play key roles in the induction of inflammation [interleukin 1β (Rubartelli et al., 1990; Andrei et al., 1999; Andrei et al., 2004; Keller et al., 2008)] and tumour-induced angiogenesis [FGF2 (Florkiewicz et al., 1995; Trudel et al., 2000; Engling et al., 2002)]. In the case of FGF2, recent evidence suggests that this mitogen follows an unconventional secretion route because it cannot be secreted by the ER-Golgi system in a functional form (Wegehingel et al., 2008). Various vesicular and non-vesicular mechanisms have been proposed to play a role in unconventional protein secretion (Nickel, 2005; Nickel and Rabouille, 2009). Vesicular mechanisms include secretory lysosomes, exosomes derived from multivesicular bodies and microvesicle shedding from cell surfaces. Interleukin 1β has been proposed to make use of at least one of these pathways (Andrei et al., 1999; MacKenzie et al., 2001; Andrei et al., 2004; Qu et al., 2007). Non-vesicular mechanisms are based on direct translocation of cytoplasmic proteins across the plasma membrane followed by deposition on cell surfaces or by release into the extracellular space (Nickel, 2005; Nickel and Rabouille, 2009). This mechanism was proposed for both FGF1 (Prudovsky et al., 2002; Prudovsky et al., 2003) and FGF2 (Schäfer et al., 2004; Zehe et al., 2006).

In terms of our knowledge about the molecular mechanisms of ER-Golgi-independent export pathways, FGF2 is so far the best-characterized example (Nickel and Seedorf, 2008). Initial sorting of FGF2 into its secretory route occurs by recruitment to the inner leaflet of plasma membranes mediated by the phosphoinositide PtdIns(4,5)P_2 (Nickel and Seedorf, 2008; Temmerman et al., 2008). The ability of FGF2 to translocate across membranes has been demonstrated in an in vitro system using affinity-purified inside-out vesicles derived from plasma membranes (Schäfer et al., 2004; Nickel, 2005). Membrane translocation of FGF2 did not depend on either ATP hydrolysis or on a membrane potential. These findings are in line with direct evidence that FGF2 secretion is driven by an extracellular trapping mechanism mediated by membrane-proximal heparan sulfate proteoglycans [HSPGs; (Zehe et al., 2006; Nickel, 2007)]. Thus, HSPGs not only function as extracellular storage sites and components of FGF2 signalling complexes but they also participate directly in FGF2 secretion (Nickel and Seedorf, 2008).

An unresolved key question is how FGF2 physically translocates across plasma membranes. Various models have been put forward such as a potential role for an FGF2 protein-conducting channel or a so far unsubstantiated ability of FGF2 to insert into membranes. In both cases, HSPG-mediated trapping of FGF2 would ensure directional transport into the extracellular space (Nickel and Seedorf, 2008). To address these options, it is of major importance to elucidate the folding state of FGF2 during membrane translocation. Initial insight came from the analysis of fusion proteins consisting of FGF2 and dihydrofolate reductase (DHFR). The DHFR domain can be stabilized with aminopterin and, therefore, certain pathways of protein translocation across membranes such as protein import into mitochondria are blocked in the presence of aminopterin or methotrexate as these mechanisms involve the unfolding of

preproteins (Eilers and Schatz, 1986; Wienhues et al., 1991). In the case of FGF2, however, fusion proteins with the DHFR domain were found to be secreted at normal rates in the presence of aminopterin (Backhaus et al., 2004). These findings were a first indication that the FGF2 export machinery might be able to translocate its substrate in a folded conformation. It could, however, not be excluded that a strong chaperone activity participates in unconventional secretion of FGF2 that is capable of unfolding the DHFR domain even in the presence of aminopterin. This was not just a hypothetical process as such a phenomenon has indeed been observed in case of protein import into chloroplasts. Although it is clear that protein translocation across the outer envelope mediated by the pore-forming TOC component Toc75 generally requires a largely unfolded state (Jarvis and Soll, 2002; Soll and Schleiff, 2004; Inaba and Schnell, 2008), it has been shown that methotrexate does not inhibit translocation of preproteins fused to DHFR (America et al., 1994). Accordingly, strong unfolding capabilities specific to the outer envelope of chloroplasts have been identified explaining the differential abilities of protein unfolding between the outer and inner membranes of chloroplasts (Guera et al., 1993; Endo et al., 1994; Walker et al., 1996; Kovacheva et al., 2007; Cline and Dabney-Smith, 2008). Thus, when studying a new pathway of protein translocation across a membrane such as FGF2 secretion, the DHFR system alone is not sufficient to claim that protein folding is maintained during membrane translocation.

In the current study, we introduce a novel experimental system that monitors the folding state of the FGF2 fusion protein during all stages of membrane translocation. The rationale of this setup was to coexpress a fusion protein of FGF2 and the FC domain of an immunoglobulin (IgG_{FC}) together with a fusion protein consisting of *Staphylococcus* protein A (SpA) and GFP. The interaction between SpA and IgG_{FC} is well known to depend on folded conformations of the two binding partners (Deisenhofer, 1981; Tashiro and Montelione, 1995; Wang et al., 1997). Using a well-characterized FGF2 secretion assay, when both fusion proteins are coexpressed within the same cells, we demonstrate that the SpA-GFP fusion protein is translocated to the cell surface in a strictly FGF2-IgG_{FC}-dependent manner. These findings show that FGF2-guided membrane translocation across plasma membranes does not depend on largely unfolded intermediates. Importantly, in addition, we demonstrate that the interaction between FGF2 and PtdIns(4,5)P_2 at the inner leaflet of plasma membranes depends on a folded conformation of FGF2. Based on our combined results, we propose that the FGF2 secretion machinery is linked to an intrinsic quality control mechanism in that recruitment to the inner leaflet depends on a folded conformation of FGF2 that is maintained during translocation across plasma membranes.

Results

An experimental system to monitor protein folding during membrane translocation

In this study, we aimed to establish an experimental system to analyze whether unfolded conformations are obligatory intermediates in FGF2 membrane translocation (Fig. 1). The rationale of this system was to coexpress two reporter molecules (one of which contains the 18 kDa form of FGF2) that interact in a folding-dependent manner. As depicted in Fig. 1A, we generated stable cell lines in which FGF2 was fused to the FC domain of an immunoglobulin (FGF2-IgG_{FC}) and, in the same cells, coexpressed a fusion protein consisting of GFP and SpA. The interaction between the IgG_{FC} domain and SpA has been characterized at atomic

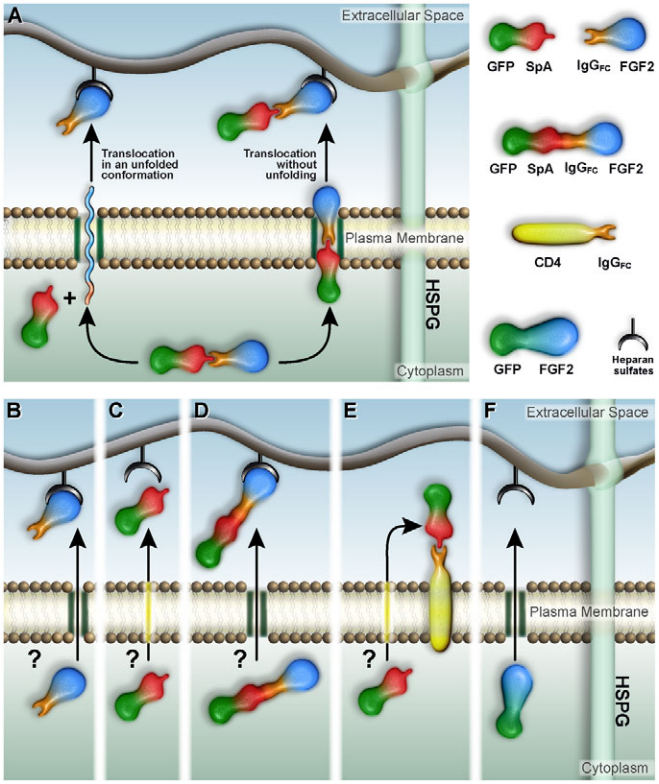

Fig. 1. Rationale of the experimental system to monitor potentially unfolded intermediates during FGF2 membrane translocation. A stable CHO cell line that coexpresses two fusion proteins, FGF2-IgG_{FC} and GFP-SpA is depicted. The two principal outcomes of the experimental setup are illustrated. (A) If GFP-SpA is retained in the cytoplasm while FGF2-IgG_{FC} is transported to the cell surface this would indicate membrane transport in a largely unfolded state resulting in a loss of interaction during membrane translocation (left-hand side). By contrast, FGF2-IgG_{FC}-dependent transport of GFP-SpA to the cell surface would demonstrate transport in a folded conformation since this scenario requires the interaction between FGF2-IgG_{FC} and GFP-SpA to be preserved during membrane translocation (right-hand side). (B-F) Controls. (B) Expression of FGF2-IgG_{FC} (to analyze whether the IgG_{FC} tag is compatible with unconventional secretion of FGF2). (C) Expression of GFP-SpA (to monitor unspecific release). (D) Expression of FGF2-IgG_{FC}-GFP-SpA (to analyze whether a fusion protein containing all four domains is compatible with unconventional secretion of FGF2). (E) Expression of both SP-IgGFC-CD4 and GFP-SpA (to monitor nonspecific release). (F) Expression of FGF2-GFP (to normalize all data using a standard FGF2 reporter molecule, the export efficiency of which has been quantified in 'secreted molecules per cell'). All fusion proteins were expressed in a doxycycline-dependent manner except SP-IgGFC-CD4 (constitutive expression).

resolution (Deisenhofer, 1981; Tashiro and Montelione, 1995) and is known to be disrupted by agents such as guanidinium hydrochloride (Wang et al., 1997), a compound that causes denaturation of folded conformations. Thus, coexport of GFP-SpA along with FGF2-IgG_{FC} as part of a non-covalent heterooligomeric complex would demonstrate that the interaction between the two fusion proteins and, therefore, folding is maintained throughout membrane translocation. By contrast, a scenario in which FGF2-IgG_{FC} would be translocated to the cell surface concomitant with retention of GFP-SpA inside cells would indicate a requirement for an unfolded intermediate in FGF2 secretion, resulting in a loss of interaction between the two fusion proteins. We used control cell lines in which either FGF2-IgG_{FC} (Fig. 1B) or GFP-SpA (Fig. 1C) were expressed alone. Another important control was a single fusion

protein consisting of all four domains (FGF2-IgG$_{FC}$-GFP-SpA; Fig. 1D) to analyze whether the relatively large size (88 kDa) of both the FGF2-IgG$_{FC}$-GFP-SpA fusion protein and the non-covalent complex of FGF2-IgG$_{FC}$ and GFP-SpA, are compatible with unconventional secretion of FGF2. Finally, we engineered a cell line in which a fusion protein of the IgG$_{FC}$ domain and the extracellular domain of the plasma membrane marker CD4 (SP-IgG$_{FC}$-CD4) was coexpressed with GFP-SpA to monitor unspecific release of GFP-SpA (Fig. 1E). A cell line expressing a fusion protein of FGF2 and GFP was used to normalize export efficiencies and to quantitatively compare the results with previous studies (Fig. 1F).

Formation of a heterooligomeric complex of FGF2-IgG$_{FC}$ and GFP-SpA

To analyze complex formation between FGF2-IgG$_{FC}$ and GFP-SpA we followed three strategies, (1) immunoprecipitation experiments, (2) gel filtration of cellular lysates and (3) binding of GFP-SpA to cell-surface-localized FGF2-IgG$_{FC}$ measured by flow cytometry. For immunoprecipitation experiments, IgG Sepharose was used to affinity-purify proteins containing a SpA domain. Both FGF2-IgG$_{FC}$-GFP-SpA (Fig. 2A; derived from the cell line depicted in Fig. 1D) and GFP-SpA (Fig. 2D; derived from the cell line depicted in Fig. 1C) could be immunoisolated employing IgG beads. By contrast, as expected, neither FGF2-IgG$_{FC}$ (Fig. 2C; derived from the cell line depicted in Fig. 1C) nor FGF2-GFP (Fig. 2E; derived from the cell line depicted in Fig. 1F) could be recovered from IgG beads but were found in the non-bound fraction. When a lysate was analyzed that was derived from cells that coexpress FGF2-IgG$_{FC}$ and GFP-SpA (Fig. 1A), both fusion proteins could be detected in the IgG-bound fraction (Fig. 2B, lane 2). Since the SpA domain used in this study contained two binding sites for IgG$_{FC}$, these results indicate that FGF2-IgG$_{FC}$ and GFP-SpA form a heterooligomeric complex in cellular lysates that can be immunopurified with IgG beads.

To verify the immunoprecipitation experiments and to analyze the amounts of GFP-SpA being engaged in heterooligomeric complexes with FGF2-IgG$_{FC}$, we conducted gel filtration of cellular lysates containing the fusion proteins indicated (Fig. 3A). The majority of the FGF2-IgG$_{FC}$-GFP-SpA fusion protein (88 kDa) was found in fractions 12-15 of a size exclusion chromatogram using a Superdex G75 column. By contrast, when expressed individually, both the FGF2-IgG$_{FC}$ and the GFP-SpA fusion protein were found in larger elution volumes with FGF2-IgG$_{FC}$ mainly appearing in fractions 15-20 and GFP-SpA in fractions 17-23. When FGF2-IgG$_{FC}$ and GFP-SpA were expressed in the same cells, subpopulations of both proteins co-migrated in fractions 12-15 (Fig. 3A). Thus, these populations of FGF2-IgG$_{FC}$ and GFP-SpA showed similar elution characteristics to the 88 kDa FGF2-IgG$_{FC}$-GFP-SpA fusion protein. We, therefore, conclude that the FGF2-IgG$_{FC}$ and GFP-SpA fusion proteins detected in fractions 12-15 are engaged in heterooligomeric complexes. Based on a quantitative comparison of the distribution of GFP-SpA across the fractions of the Superdex G75 size exclusion chromatogram, we found about 15% of GFP-SpA in complexes with FGF2-IgG$_{FC}$ at steady-state. A significantly larger fraction of FGF2-IgG$_{FC}$ was found in fractions 12-15 because of a lower level of expression of FGF2-IgG$_{FC}$ compared with GFP-SpA, i.e. most of the FGF2-IgG$_{FC}$ fusion proteins were engaged in complexes whereas excess amounts of GFP-SpA remained monomeric.

Finally, we analyzed binding of GFP-SpA to cell-surface-localized FGF2-IgG$_{FC}$ and SP-IgG$_{FC}$-CD4 (Fig. 3B). We used stable cell lines that expressed either FGF2-IgG$_{FC}$ or SP-IgG$_{FC}$-CD4 and

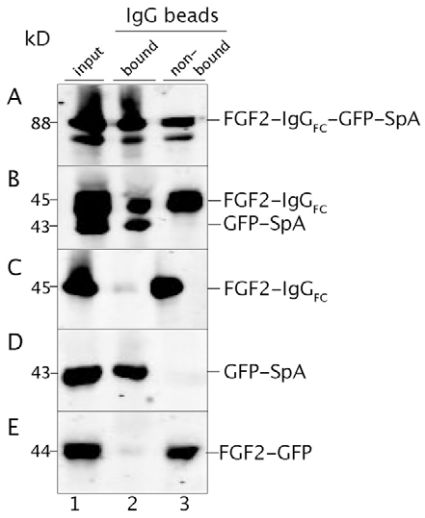

Fig. 2. Formation of a heterodimeric complex between FGF2-IgG$_{FC}$ and GFP-SpA as demonstrated by immunoprecipitation experiments. Cells expressing the fusion proteins indicated were used to prepare lysates that were incubated with IgG Sepharose beads. Following extensive washing, bound and unbound fractions were analyzed by SDS-PAGE and western blotting. Lysates to be analyzed were derived from cells either expressing FGF2-IgG$_{FC}$-GFP-SpA (A), coexpressing FGF2-IgG$_{FC}$ and GFP-SpA (B), or expressing the fusion proteins FGF2-IgG$_{FC}$ (C), GFP-SpA (D) or FGF2-GFP (E). Input: 10% (lane 1); bound fraction: 33% (lane 2); non-bound material: 7.5% (lane 3). For antigen detection, monoclonal mouse anti-FGF2 antibodies (Sigma-Aldrich F6162) and Alexa-Fluor-680-coupled goat anti-mouse antibodies (Molecular Probes) were used.

added various amounts of exogenous GFP-SpA at 4°C. Binding of GFP-SpA to cell-surface-exposed FGF2-IgG$_{FC}$ and SP-IgG$_{FC}$-CD4, was quantified by flow cytometry using affinity-purified anti-GFP antibodies (Fig. 3B). These experiments showed that binding of GFP-SpA to both SP-IgG$_{FC}$-CD4 (Fig. 3B, light green bars) and FGF2-IgG$_{FC}$ (Fig. 3B, dark green bars) was readily detectable. Thus, under these experimental conditions, a heterooligomeric complex of FGF2-IgG$_{FC}$ and GFP-SpA could be detected on cell surfaces and, therefore, when SP-IgG$_{FC}$-CD4 and GFP-SpA are coexpressed in the same cells (as depicted in Fig. 1E), a potentially occurring nonspecific release of GFP-SpA can be monitored by flow cytometry experiments.

GFP-SpA is transported to cell surfaces as part of a heterooligomeric complex with FGF2-IgG$_{FC}$

To analyze the potential ability of FGF2-IgG$_{FC}$ to export GFP-SpA as part of a heterooligomeric complex we used cell lines expressing the fusion proteins depicted in Fig. 1A,C,D,E,F. The rationale was to measure GFP fluorescence to monitor total expression levels (Fig. 4A, green bars) combined with the specific detection of cell-surface-exposed populations measured by antibody staining and quantification using a well-characterized flow cytometry assay (Engling et al., 2002; Seelenmeyer et al., 2005; Zehe et al., 2006; Temmerman et al., 2008). Cell-surface-localized fusion proteins containing IgG$_{FC}$ domains were detected with anti-mouse IgG$_{FC}$ secondary antibodies (Fig. 4A, red bars). Fusion proteins containing GFP were detected with affinity-purified anti-GFP antibodies (Fig. 4A, yellow bars). All data shown in Fig. 4A were normalized using a cell line expressing the fusion protein containing all four domains (FGF2-IgG$_{FC}$-GFP-SpA; Fig. 1D). Of note, all measurements

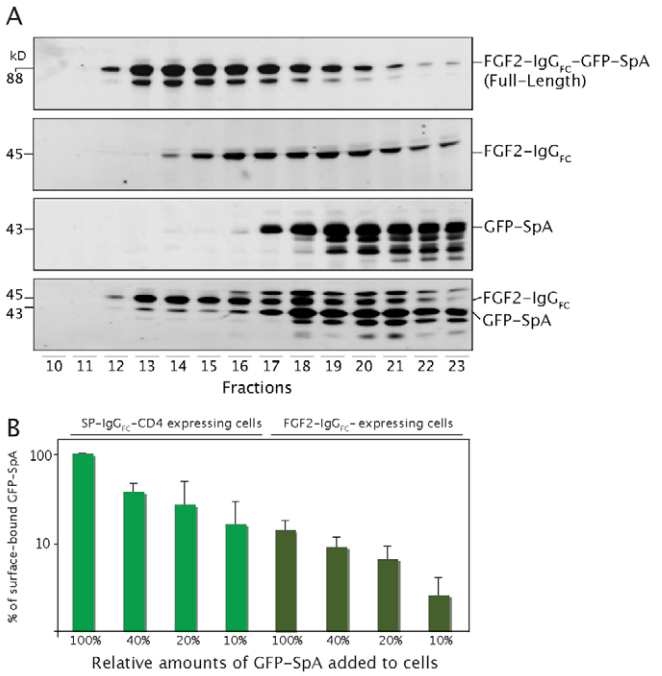

Fig. 3. Analysis of complex formation between FGF2-IgG$_{FC}$ and GFP-SpA. (A) Size exclusion chromatography. Cellular lysates (3 mg total protein) containing the fusion proteins indicated were applied to a Superdex G75 column (24 ml bed volume). Fractions (300 µl) were collected and 5% of each sample were analyzed by SDS-PAGE and western blotting. Size standards: ovalbumin (44 kDa) was recovered from fractions 19-23. For antigen detection, monoclonal mouse anti-FGF2 antibodies (Sigma-Aldrich F6162) and affinity-purified anti-GFP antibodies (Engling et al., 2002), were used. Alexa-Fluor-680-coupled goat anti-mouse and anti-rabbit antibodies (Molecular Probes) were used as secondary antibodies. (B) FACS analysis of GFP-SpA binding to cell surfaces presenting SP-IgG$_{FC}$-CD4 (light-green bars) and FGF2-IgG$_{FC}$ (dark-green bars). Various amounts of GFP-SpA (100, 40, 20 and 10% of material derived from 7×10^5 cells expressing GFP-SpA) were added to 7×10^5 target cells expressing either FGF2-IgG$_{FC}$ or SP-IgG$_{FC}$-CD4. Cell-surface-bound GFP-SpA was quantified by flow cytometry (n=4). The data shown were corrected for background signals detected with cells that did not express IgG$_{FC}$ fusion proteins.

shown in Fig. 4A (green bars) were conducted under conditions where the GFP-SpA fusion proteins were expressed at similar levels, i.e. the corresponding cell surface signals are directly comparable. When a standard FGF2-GFP fusion protein (Zehe et al., 2006; Temmerman et al., 2008) was compared with FGF2-IgG$_{FC}$-GFP-SpA we found a twofold increase in cell surface expression of FGF2-GFP (45 kDa) suggesting that the relatively large size of the FGF2-IgG$_{FC}$-GFP-SpA fusion protein (88 kDa) reduces export efficiency by about 50%. As expected, the FGF2-GFP fusion protein was not detectable with anti-mouse IgG$_{FC}$ secondary antibodies as it does not contain an IgG$_{FC}$ domain (Fig. 4A, red bars). When we analyzed a cell line expressing both FGF2-IgG$_{FC}$ and GFP-SpA (Fig. 1A), we observed a cell surface signal for both FGF2-IgG$_{FC}$ (Fig. 4B, red bars) and GFP-SpA (Fig. 4A, yellow bars). The observed cell surface signal for GFP-SpA at about 20% export efficiency compared with FGF2-IgG$_{FC}$-GFP-SpA demonstrates export of GFP-SpA as part of a heterooligomeric complex with FGF2-IgG$_{FC}$. This is because the corresponding measurements of the cell lines depicted in Fig. 1C (expression of GFP-SpA alone) and Fig. 1E (expression of SP-IgG$_{FC}$-CD4 plus GFP-SpA), respectively, revealed that, in the absence of FGF2-IgG$_{FC}$, GFP-SpA is not exported from cells. This conclusion is based on the absence of GFP-SpA on both cell surfaces (Fig. 4A) and in cellular supernatants (Fig. 4B).

The data discussed above are consistent with the findings documented in Figs 2 and 3. Since we found only about 15% of GFP-SpA being complexed with FGF2-IgG$_{FC}$ in lysates derived from cells depicted in Fig. 1A and since FGF2-IgG$_{FC}$ was found to be expressed at a about three times lower expression level compared with FGF2-IgG$_{FC}$-GFP-SpA (data not shown), our data indicate that the heterooligomeric complex of these two proteins is exported at least at an equal efficiency compared to the FGF2-IgG$_{FC}$-GFP-SpA fusion protein in which all four domains are covalently linked. In terms of absolute efficiency, the reference cell line used in Fig. 4 has previously been shown to export FGF2-GFP at a rate that, under steady-state conditions, results in 1.25×10^6 FGF2-GFP molecules on the cell surface of a single cell (Zehe et al., 2006). Thus, based on the quantifications shown in Fig. 4A, the FGF2-IgG$_{FC}$ fusion protein is capable of exporting GFP-SpA to the cell surface at an efficiency of about 125,000 molecules per cell at steady state. Export of GFP-SpA is strictly dependent on coexpression of FGF2-IgG$_{FC}$ and, therefore, is not related to nonspecific release. This conclusion is in line with the fact that nonspecific release of GFP-SpA is undetectable in a cell line expressing SP-IgG$_{FC}$-CD4 (Fig. 1E, Fig. 4A), a sensor of extracellular fusion proteins containing SpA domains.

PtdIns(4,5)P_2-dependent membrane recruitment of FGF2 depends on folding

The observation that FGF2 secretion does not involve unfolding as an obligatory intermediate prompted us to address the question of whether an intrinsic quality control mechanism may be part of the unconventional export pathway of FGF2. In previous work, we demonstrated that PtdIns(4,5)P_2-dependent recruitment to the inner leaflet of plasma membranes represents the entry point of the FGF2 export pathway (Temmerman et al., 2008; Nickel and Rabouille, 2009). We, therefore, investigated whether the interaction with PtdIns(4,5)P_2 depends on a folded conformation of FGF2. Using a previously established assay to quantify interactions between soluble proteins and membrane lipids (Temmerman et al., 2008; Temmerman and Nickel, 2009), we demonstrate that the interaction between PtdIns(4,5)P_2 and FGF2 is disrupted following FGF2 unfolding in the presence of urea (Fig. 5C). We pre-incubated FGF2-GFP with urea at the concentrations indicated followed by incubation with PtdIns(4,5)P_2-containing liposomes (Fig. 5C, blue bars). For all experimental conditions, as a control, we also analyzed FGF2-GFP binding to liposomes lacking PtdIns(4,5)P_2 (Fig. 5C, red bars). The amounts of FGF2-GFP bound to liposomes under the conditions indicated were quantified by flow cytometry. The results shown in Fig. 5C demonstrate that the interaction between FGF2 and PtdIns(4,5)P_2-containing liposomes is lost when FGF2-GFP was incubated in the presence of urea. A urea concentration of 2 M was sufficient to block binding by about 85%.

To analyze whether FGF2 unfolds in the presence of urea in a concentration range of 2-4 M we monitored the folding state of FGF2 by far-UV circular dichroism (CD) spectroscopy. We first determined the CD spectrum of native FGF2. As shown in Fig. 5A (black line) the CD spectrum of FGF2 has a maximum at 228 nm and a minimum at 201 nm, indicative of a protein that contains β-sheets, β-turns and random coil but no α-helices. These findings are consistent with the known crystal structure of FGF2 (Kastrup et al., 1997). Next we determined the CD spectra for FGF2 at

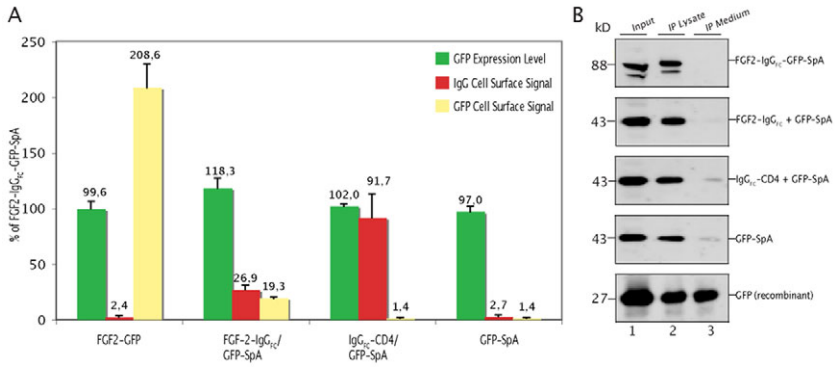

Fig. 4. FGF2-IgG$_{FC}$-dependent secretion of GFP-SpA. (A) Analysis of cell surface exposure of the fusion proteins indicated using flow cytometry (n=4). GFP expression level is shown as green bars; cell surface populations detected by anti-IgG$_{FC}$ antibodies is shown as red bars; cell surface populations detected by anti-GFP antibodies is shown as yellow bars. (B) Biochemical analysis of lysates and cell-culture media from cells expressing the fusion proteins indicated. GFP fusion proteins were immunoprecipitated using affinity-purified anti-GFP antibodies coupled to protein A beads. 25% of input (lane 1), immunoprecipitated material from lysates (lane 2) and immunoprecipitated material from media (lane 3) were analyzed by SDS-PAGE and western blotting. Recombinant GFP was used as a positive control for immunoprecipitation experiments. For antigen detection, monoclonal mouse anti-FGF2 antibodies (Sigma-Aldrich F6162) and Alexa-Fluor-680-coupled goat anti-mouse antibodies (Molecular Probes) were used.

different concentrations of urea (Fig. 5A). Since urea absorbs below 210 nm we plotted the relative elipticity at 228 nm versus the urea concentration. As can be seen in Fig. 5B, the relative elipticity at 228 nm decreases with increasing urea concentrations according to a two-state unfolding transition. At 3-4 M urea, the lower plateau of the unfolding transition was reached. Our data demonstrate that FGF2 unfolding strongly correlates with the loss of its ability to bind to PtdIns(4,5)P_2-containing liposomes. As shown in Fig. 5D,E, urea-mediated unfolding of FGF2 also blocks FGF2 binding to heparin, an interaction that is known to involved folded FGF2 as well. However, as opposed to binding to PtdIns(4,5)P_2-containing liposomes, residual binding of FGF2 to heparin could still be observed at 4 M urea. These findings suggest that, albeit with much lower efficiency, FGF2 can interact with heparin in an unfolded state. Our observations are consistent with other cases of proteins that bind to PtdIns(4,5)P_2 such as those containing a PH domain, since crystal structures have been obtained that demonstrate a defined binding pocket for the headgroup of PtdIns(4,5)P_2, InsP_3 (Ferguson et al., 1995; Lemmon, 2008). We conclude that membrane recruitment by PtdIns(4,5)P_2 at the inner leaflet of plasma membranes depends on the folding state of FGF2 and, therefore, largely unfolded or misfolded FGF2 molecules are likely to be prevented from entering the FGF2 export pathway.

Discussion

Protein translocation across membranes in many cases requires substrates that are largely unfolded as is the case in Sec61-dependent protein translocation across the membrane of the endoplasmic reticulum (Osborne et al., 2005) or in protein import into mitochondria (Rehling et al., 2004; Neupert and Herrmann, 2007). Rare exceptions of this situation appear to be protein export in prokaryotes mediated by the twin arginine system (Lee et al., 2006; Sargent, 2007) and protein import into peroxisomes (Leon et al., 2006). In the case of the unconventional secretory pathway of FGF2 we have previously shown that FGF2 as a fusion protein with the DHFR domain can translocate across plasma membranes in the presence of aminopterin (Backhaus et al., 2004), a drug that prevents unfolding of the DHFR domain (Eilers and Schatz, 1986; Wienhues et al., 1991). Although these findings were interpreted as initial evidence for FGF2 membrane translocation in a folded conformation, a lack of a transport block observed with DHFR fusion proteins in the presence of aminopterin alone represents negative evidence and, therefore, does not appear to be sufficient to conclude that the protein remains folded during all stages of membrane translocation. Furthermore, examples exist of membrane translocation in a largely unfolded state, such as protein transport across the outer envelope of chloroplasts; however, this process was shown to be compatible with DHFR fusion proteins in the presence of stabilizing ligands (Guera et al., 1993; America et al., 1994; Endo et al., 1994; Walker et al., 1996; Jarvis and Soll, 2002; Soll and Schleiff, 2004; Kovacheva et al., 2007; Cline and Dabney-Smith, 2008; Inaba and Schnell, 2008). Therefore, in the current study, we introduced a novel experimental system to investigate whether unfolded FGF2 molecules are obligatory intermediates for membrane translocation as part of the unconventional secretory pathway of FGF2. The system is based on a heterooligomeric complex of two fusion proteins, FGF2-IgG$_{FC}$ and GFP-SpA, an interaction known to depend on folded conformations of the two interaction partners (Deisenhofer, 1981; Tashiro and Montelione, 1995; Wang et al., 1997). We find that GFP-SpA is efficiently exported from these cells in a strictly FGF2-IgG$_{FC}$-dependent manner. These data unequivocally demonstrate that there is no requirement for an unfolded intermediate in FGF2 membrane translocation, i.e. the FGF2 fusion proteins used are capable of physically traversing the plasma membrane in a folded conformation.

Our results have important implications both for the molecular mechanism of FGF2 membrane translocation and for quality control aspects of this pathway. Three models have been proposed for FGF2 translocation across plasma membranes (Nickel and Seedorf, 2008). The first involves a classical protein-conducting channel such as Sec61 at the endoplasmic reticulum, however, in the light of the current data, this appears to be unlikely as this type of membrane translocation is restricted to unfolded substrates (Osborne et al., 2005; Neupert and Herrmann, 2007). Two other models of FGF2 membrane translocation propose a potential ability of FGF2 to transiently insert into membranes (Nickel and Seedorf, 2008). This process is likely to be facilitated by resident proteins of plasma membranes. For both models, it was suggested that PtdIns(4,5)P_2-dependent recruitment of FGF2 at the inner leaflet of plasma

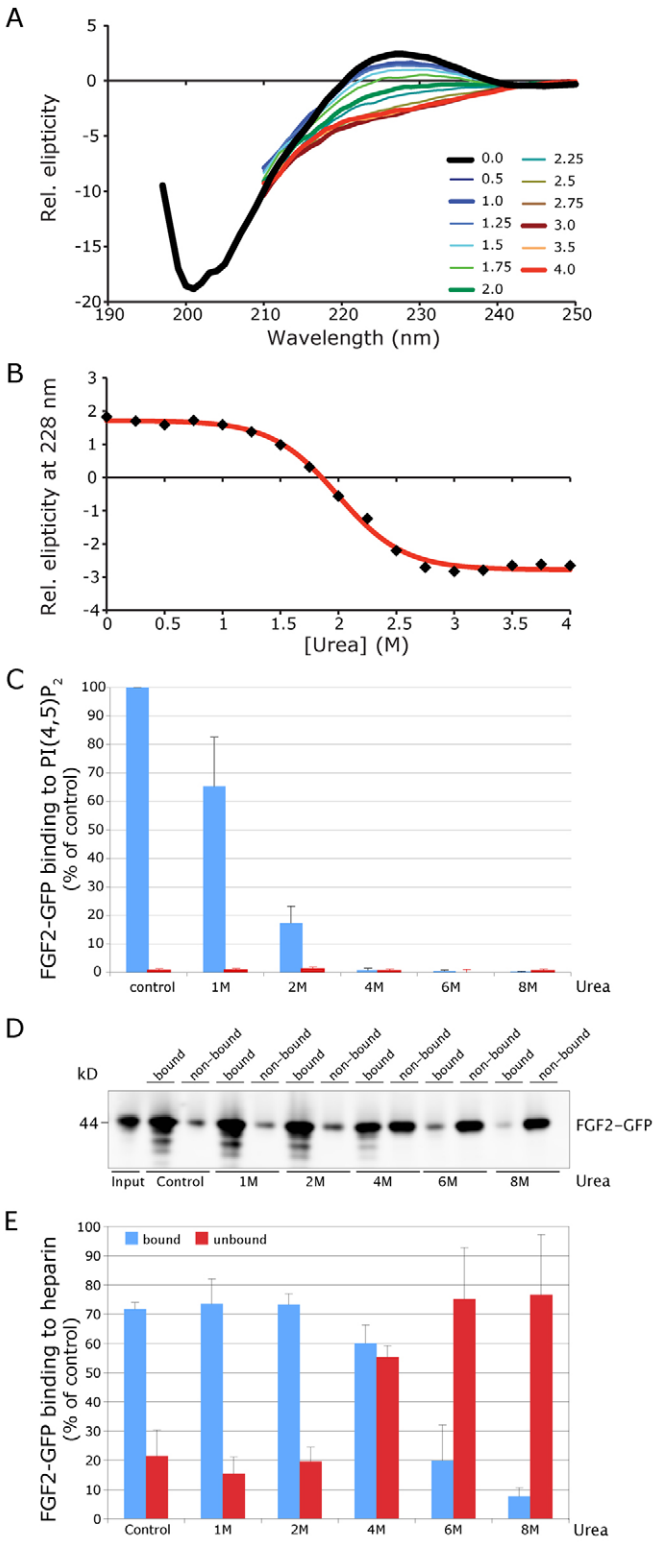

Fig. 5. Binding to PtdIns(4,5)P_2 depends on a folded conformation of FGF2. (A) Analysis of the folding state of FGF2 in the presence of urea. Far-UV circular dichroism spectra of FGF2 in the absence of urea (black line) and in the presence of increasing concentrations of urea as indicated. (B) Denaturation of FGF2 by urea followed by CD spectroscopy at 228 nm. The red line represents a data fit using the equation for chemical unfolding (see Material and Methods). An unfolding midpoint at 1.997±0.003 M urea was calculated as the average of two independent experiments. (C) Binding of a FGF2-GFP fusion protein to PtdIns(4,5)P_2-containing liposomes was quantified by flow cytometry as described previously (Temmerman et al., 2008; Temmerman and Nickel, 2009). FGF2-GFP was pretreated with a buffer containing urea, at the concentrations indicated, followed by incubation in the presence of PtdIns(4,5)P_2-containing liposomes (blue bars). Control experiments were performed with liposomes lacking PtdIns(4,5)P_2 (red bars). Data are expressed as the percentage of FGF2-GFP binding to PtdIns(4,5)P_2-containing liposomes measured without pre-treatment with urea. Error bars represent standard deviations (n=5). (D) Binding of a FGF2-GFP fusion protein to heparin beads. FGF2-GFP was pretreated with a buffer containing urea at the concentrations indicated. Binding experiments using heparin beads were conducted as described previously (Zehe et al., 2006). Both bound and unbound material was analyzed by SDS-PAGE and western blotting using anti-GFP antibodies. (E) Quantification of the western blot signals shown in D using a LI-COR infrared imaging system. Error bars represent standard deviations (n=3).

membranes (Temmerman et al., 2008) might represent a crucial initial step in membrane insertion followed by translocation into the extracellular space mediated by the HSPG trapping mechanism (Zehe et al., 2006; Nickel, 2007). One model proposed that conformational changes in FGF2 may mediate membrane insertion with direct contact to the hydrophobic core of the bilayer (Nickel and Seedorf, 2008), however, the general compatibility of FGF2 secretion with various kinds of tags such as GFP (Engling et al., 2002; Zehe et al., 2006), DHFR (Backhaus et al., 2004) and IgG_{FC} (this study) makes it rather unlikely that all of these FGF2 fusion proteins could acquire a conformation that would allow membrane insertion with contact to the hydrophobic core.

The second model of membrane insertion of FGF2 is based on the opening of a hydrophilic pore, the inner surface of which is lined up with the headgroup of the phosphoinositide PtdIns(4,5)P_2 (Nickel and Seedorf, 2008). It was speculated that the interaction with PtdIns(4,5)P_2 causes FGF2 to oligomerize, resulting in the formation of a multivalent complex. Depending on the geometry of the PtdIns(4,5)P_2 binding sites, an oligomeric FGF2 complex might be able to open up a hydrophilic pore concomitant with self-insertion into the membrane (Nickel and Seedorf, 2008). Externalization would follow by HSPG-dependent trapping in the extracellular space (Zehe et al., 2006; Nickel, 2007). This potential mechanism of FGF2 membrane translocation appears to be consistent with the results of this study as it is likely to be compatible with both a variety of FGF2 fusion proteins and with FGF2 export in a folded conformation. Therefore, it will be a major challenge for future studies to experimentally address the potential ability of FGF2 to self-insert into membranes in a PtdIns(4,5)P_2-dependent manner.

Another important implication of this study is an intrinsic mechanism of quality control as part of the FGF2 secretion pathway. Since FGF2 secretion occurs by direct translocation across plasma membranes (Schäfer et al., 2004; Nickel, 2005; Zehe et al., 2006; Nickel, 2007), a potential requirement for an unfolded intermediate in FGF2 secretion would ultimately result in the secretion of non-functional molecules in terms of biological activity. The discovery that membrane-proximal HSPGs form an extracellular trap that is essential for FGF2 secretion (Zehe et al., 2006; Nickel, 2007) was a first hint that FGF2 appears on the extracellular side of the plasma membrane in a folded state. This is because efficient interactions mediated by HSPGs depend on a folded conformation of FGF2 (Faham et al., 1996; Faham et al., 1998; Raman et al., 2003). Similarly, the interaction of FGF2 with PtdIns(4,5)P_2 is likely to depend on a specific binding pocket, as demonstrated for other PtdIns(4,5)P_2-

binding proteins such as those containing PH domains (Ferguson et al., 1995; Lemmon, 2008). However, the interaction between FGF2 and PtdIns(4,5)P_2 is mediated by a different motif, a C-terminal basic cluster, for which a structural analysis at atomic resolution is so far not available. In the current study, we demonstrate that the interaction between FGF2 and PtdIns(4,5)P_2 depends on a folded conformation as it is completely lost upon urea-mediated unfolding of FGF2. Thus, both PtdIns(4,5)P_2-dependent recruitment to the inner leaflet and HSPG-dependent translocation to cell surfaces requires proper folding of FGF2. Therefore, combined with our finding that unfolded intermediates of FGF2 are not required for membrane translocation, we propose that membrane translocation of FGF2 is not only compatible with a folded conformation but indeed depends on it to ensure secretion of biologically active molecules.

Materials and Methods

Generation of stable cell lines expressing various fusion proteins in a doxycycline-dependent manner

The constructs encoding the fusion proteins depicted in Fig. 1 were either used with the vector pRevTRE2 (doxycycline-dependent expression of FGF2-IgG_{FC}, GFP-SpA, FGF2-IgG_{FC}-GFP-SpA, FGF2-GFP) or pFB (constitutive expression of SP-IgGFc-CD4). 'SP' indicates a conventional signal peptide that directs SP-IgGFc-CD4 to the classical secretory pathway and promotes cell surface expression. The SpA domain used in this study was based on two B domains with a total of 137 amino acids generating two binding sites for IgG_{FC} (Popplewell et al., 1991; Bottomley et al., 1994). Following retroviral transduction, stable CHO cell lines were isolated by FACS sorting as described previously (Engling et al., 2002).

Analysis of complex formation of FGF2-IgG_{FC} and GFP-SpA by immunoprecipitation experiments

CHO cells expressing the fusion proteins indicated were grown in culture dishes (10 cm diameter) for 36 hours in the presence of doxycycline (1 μg/ml) resulting in a confluency of about 80%. Cells were detached with PBS plus 0.5 mM EDTA and solubilized in 1% Triton X-100–PBS containing a protease inhibitor cocktail (Roche). Following incubation for 15 minutes at room temperature with gentle stirring every 5 minutes, cell lysates were cleared of cell debris by centrifugation at 20,000 ***g*** for 10 minutes at 4°C. IgG Sepharose beads (GE Healthcare) were used to immunoisolate fusion proteins containing a SpA domain. All further centrifugation steps were performed at 800 ***g*** for 5 minutes at 4°C. Defined amounts of total lysates were incubated with 50 μl IgG Sepharose beads for 2 hours at 4°C. After extensive washing with buffer (25 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA), bound proteins were eluted with SDS sample buffer and separated by SDS-PAGE using 4-12% gradient gels. Western blotting was performed using anti-FGF2 (F6162; Sigma-Aldrich) and anti-mouse IgG antibodies coupled with Alexa Fluor 680 (Molecular Probes).

Analysis of complex formation of FGF2-IgG_{FC} and GFP-SpA by size-exclusion chromatography

CHO cells expressing the fusion proteins indicated were grown as described above. Cells were detached with PBS plus 0.5 mM EDTA supplemented with a protease inhibitor cocktail (Roche). Homogenization was achieved by three freeze-thaw cycles followed by sonication. The resulting homogenate was subjected to low speed (10,000 ***g***, 10 minutes, 4°C) and high speed (100,000 ***g***, 1 hour, 4°C) centrifugations resulting in a clear protein solution with organelles and membrane debris being removed. Cell-free supernatants were separated on a Superdex G75 gel filtration column (24 ml bed volume) employing a GE ÄKTA chromatography system. 3 mg of total protein were applied in each individual run, and fractions of 300 μl were collected. The material from each fraction (5%) was subjected to SDS-PAGE and western blotting. The amount of GFP-SpA present in a complex with FGF-2-IgG_{Fc} at steady-state (fractions 12-15; Fig. 3A) was calculated as the percentage of the total GFP-SpA signal distributed across the chromatography column (fractions 12-23; Fig. 3A). Quantification was carried out using an Odyssey infrared imaging system (LI-COR Biosciences). A 44 kDa marker (ovalbumin; Bio-Rad) was used as a standard and found to elute with GFP-SpA in fractions 19-23.

Quantification of GFP-SpA binding to cells with either FGF2-IgG_{FC} or SP-IgG_{FC}-CD4 on their cell surfaces

Cell-free supernatants from cells expressing GFP-SpA were prepared as described above. Various percentages of material derived from 7×10^5 cells were added to 7×10^5 target cells expressing either FGF2-IgG_{FC} or SP-IgG_{FC}-CD4. Following incubation for 1 hour at 4°C, binding of GFP-SpA to either FGF2-IgG_{FC} or SP-IgG_{FC}-CD4 was quantified by flow cytometry using affinity-purified rabbit anti-GFP and anti-rabbit IgG antibodies coupled to allophycocyanine (Molecular Probes). Background values of SpA-GFP were determined by using target cells that do not express FGF2-IgG_{FC} or SP-IgG_{FC}-CD4. The relative amounts of FGF2-IgG_{FC} and SP-IgG_{FC}-CD4, were quantified using anti-mouse IgG antibodies coupled to allophycocyanine (Molecular Probes). The latter experiments revealed about three times more SP-IgG_{FC}-CD4 than FGF2-IgG_{FC}on cell surfaces.

Quantitative analysis of the secretion of heterodimeric complexes made of FGF2-IgG_{FC} and GFP-SpA

CHO cells expressing the fusion proteins indicated were grown in 12-well plates for 36 hours in the presence of doxycycline (1 μg/ml) resulting in a confluency of about 70%. Doxycyline concentrations were titrated to obtain conditions under which the GFP-containing fusion proteins in all cell lines were expressed at similar levels. Following detachment, using cell dissociation buffer (Life Technologies), and extensive washing with PBS, cells were decorated with either affinity-purified rabbit anti-GFP and anti-rabbit IgG antibodies coupled to allophycocyanine (1 hour, 4°C) or with anti-mouse IgG antibodies coupled to allophycocyanine (30 minutes, 4°C). The relative amounts of cell-surface-localized GFP- and IgG_{FC}-containing fusion proteins were quantified using a BD FACSCalibur flow cytometer. Cells were stained with propidium iodide and damaged cells were excluded from the analysis.

Detection of soluble GFP fusion proteins in cellular supernatants

CHO cells expressing the fusion proteins indicated were grown as described above. Following removal of the cell-culture supernatants, cellular lysates were prepared as described above. Cell-culture medium was centrifuged (500 ***g***, 20 minutes, 4°C) to remove damaged cells and membrane debris. GFP-containing fusion proteins were immunoprecipitated from both lysates and cell-culture supernatants using affinity-purified rabbit anti-GFP antibodies coupled to protein A beads (GE Healthcare). Samples were analyzed by SDS-PAGE and western blotting using monoclonal anti-GFP antibodies (Clontech).

Interaction studies to probe FGF-2 binding to PtdIns(4,5)P_2-containing liposomes and heparin-coated beads

Plasma membrane-like, large unilamellar liposomes were generated employing a size-extrusion approach as described previously (Temmerman and Nickel, 2009). The lipid composition was 50 mol% cholesterol, 12.5 mol% phosphatidylcholine, 9 mol% phosphatidylethanolamine, 5 mol% phosphatidylserine, 5 mol% phosphatidylinositol, 12.5 mol% sphingomyelin and 5 mol% phosphatidylinositol-4,5-bisphosphate. For control experiments, phosphatidylinositol-4,5-bisphosphate was replaced by phosphatidylcholine. The final liposome-containing solution was obtained in reconstitution buffer (150 mM KCl and 25 mM Hepes, pH 7.4) supplemented with 10% (w/v) sucrose. Liposomal binding assays were performed as described previously (Temmerman and Nickel, 2009). Briefly, FGF2-GFP (100 μg/ml) was pre-incubated for 1 hour with various concentrations of urea (in reconstitution buffer) and subsequently added to liposomes (total lipid concentration: 1 mM) for 3 hours. Following incubation with protein, liposomes were washed with the corresponding urea concentration (up to 2 M) in reconstitution buffer, and liposome-bound protein was measured using flow cytometry (Temmerman and Nickel, 2009). Samples with equimolar amounts of GFP were processed in parallel and served to quantify background signals. Data were normalized by setting the fluorescence signal of a sample incubated in the absence of urea to 100%. As an additional control we analyzed whether GFP becomes unfolded in the presence of urea, resulting in a loss of fluorescence. Following incubations for 2 hours in 8 M urea, we observed a reduction in GFP fluorescence of about 10%. Incubations for 2 hours in 6 M urea revealed no significant reduction in GFP fluorescence under these experimental conditions.

FGF2-GFP binding to heparin beads was assayed as described previously (Zehe et al., 2006). Per binding experiment, 10 μg of FGF2-GFP was incubated with 4 μl of heaprin beads (GE Healthcare) in the absence or presence of urea as indicated. For each experimental condition, input, bound and unbound material (2% each) were analyzed by SDS-PAGE and western blotting using anti-GFP antibodies.

Circular dichroism spectroscopy

CD spectra were measured in a Jasco J-750 spectropolarimeter at 0.1 cm path length and 25°C using 10 μM FGF2 in 50 mM $K_xH_yPO_4$ pH 7.5 and urea at the concentrations indicated. The following equation was fitted to the data:

$$\Theta = \frac{\Theta_F + \Theta_U \cdot e^{\frac{m\cdot\left([urea]-[urea]^{50\%}\right)}{RT}}}{1 + e^{\frac{m\cdot\left([urea]-[urea]^{50\%}\right)}{RT}}},$$

where Θ_F and Θ_U are the relative elipticity of folded and unfolded FGF2, $[urea]^{50\%}$ is the urea concentration at which 50% of the molecules are unfolded and 50% native, m is the coefficient of proportionality, R the gas constant and T the absolute temperature.

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