Biosynthetic FGF-2 is targeted to non-lipid raft microdomains following translocation to the extracellular surface of CHO cells

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Summary

Basic fibroblast growth factor (FGF-2) is a secretory protein that lacks a signal peptide. Consistently, FGF-2 has been shown to be secreted by an ER-Golgi-independent mechanism; however, the machinery mediating this process remains to be established at the molecular level. Here we introduce a novel experimental system based on flow cytometry that allows the quantitative assessment of non-classical FGF-2 secretion in living cells. Stable cell lines have been created by retroviral transduction that express various kinds of FGF-2-GFP fusion proteins in a doxicyclin-dependent manner. Following induction of protein expression, biosynthetic FGF-2-GFP is shown to translocate to the outer surface of the plasma membrane as determined by both fluorescence activated cell sorting (FACS) and confocal microscopy. Both N- and C-terminal GFP tagging of FGF-2 is compatible with FGF-2 export, which is shown to occur in a controlled fashion rather than through unspecific release. The experimental system described has strong implications for the identification of both FGF-2 secretion inhibitors and molecular components involved in FGF-2 secretion.

In the second part of this study we made use of the FGF-2 export system described to analyze the fate of biosynthetic FGF-2-GFP following export to the extracellular space. We find that secreted FGF-2 fusion proteins accumulate in large heparan sulfate proteoglycan (HSPG)-containing protein clusters on the extracellular surface of the plasma membrane. These microdomains are shown to be distinct from caveolae-like lipid rafts known to play a role in FGF-2-mediated signal transduction. Since CHO cells lack FGF high-affinity receptors (FGFRs), it can be concluded that FGFRs mediate the targeting of FGF-2 to lipid rafts. Consistently, FGF-2-GFP-secreting CHO cells do not exhibit increased proliferation activity. Externalization and deposition of biosynthetic FGF-2 in HSPG-containing protein clusters are independent processes, as a soluble secreted intermediate was demonstrated. The balance between intracellular FGF-2 and HSPG-bound secreted FGF-2 is shown not to be controlled by the availability of cell surface HSPGs, indicating that the FGF-2 secretion machinery itself is rate-limiting.

Key words: Non-conventional protein secretion, Non-classical protein export, Membrane translocation, Extracellular FGF-2, FGF-2 signalling, FGF-2 microdomains

Introduction

Proteins destined for the classical pathway of eukaryotic secretion typically contain an N-terminal signal peptide (Walter et al., 1982) that mediates translocation into the lumen of the endoplasmic reticulum (ER) followed by ER/Golgi-dependent vesicular transport to the cell surface (Rothman and Wieland, 1996; Schatz and Dobberstein, 1996; Nickel et al., 1998; Mellman and Warren, 2000). By contrast, several proteins such as angiogenic growth factors [fibroblast growth factor (FGF) 1 and 2 (Jackson et al., 1992; Mignatti et al., 1992; Florkiewicz et al., 1995; Jackson et al., 1995)], cytokines such as interleukin 1β (IL1β) and thioredoxin (Rubartelli et al., 1990; Rubartelli and Sitia, 1991; Rubartelli et al., 1992), lectins of the extracellular matrix [the protein family of the galectins (Cooper and Barondes, 1990; Cho and Cummings, 1995b; Cho and Cummings, 1995a; Cleves et al., 1996; Mehul and Hughes, 1997; Menon and Hughes, 1999)], viral proteins such as Herpes simplex tegument protein VP22 (Elliott and O'Hare, 1997) as well as other molecules (Ensoli et al., 1993; Sloan et al., 1994; Denny et al., 2000; Lecellier et al., 2002) have been identified as secretory proteins but they lack a signal peptide (for a review, see Hughes, 1999). Consistently, these proteins are characterized by: (1) not being found in subcellular compartments that belong to the ER-Golgi-dependent secretory pathway; (2) not being glycosylated despite bearing multiple consensus sequences for this ER-Golgi-dependent modification; and (3) not being blocked with regard to secretion by inhibitors of the classical secretory pathway such as brefeldin A and monensin (Cleves, 1997; Hughes, 1999). Thus, a non-conventional mechanism of protein secretion has been postulated (Cleves, 1997; Hughes, 1999); however, the

molecular machinery mediating this process remains to be identified at the molecular level.

In case of FGF-2, the sodium potassium ATPase (Na-K-ATPase) has been proposed to play a role in FGF-2 export from cells. This conclusion is based on the observation that FGF-2 export is partially inhibited in the presence of ouabain (Florkiewicz et al., 1998), a drug known to inhibit the Na-K-ATPase (Jorgensen and Pedersen, 2001). These results were further substantiated by data demonstrating that FGF-2 export in the presence of ouabain recovers when an ouabain-resistant mutant of the α-subunit of the Na-K-ATPase is expressed (Dahl et al., 2000). However, it remains unclear as to whether the Na-K-ATPase plays a direct role in the translocation mechanism of FGF-2 that results in the release into the extracellular space.

While export of FGF-1 follows a non-conventional route as well, differences from FGF-2 export have been reported such as increased secretion of FGF-1 following heat shock treatment (Jackson et al., 1992; Jackson et al., 1995). S100A13, a member of the S100 protein family of Ca^{2+}-binding proteins (Donato, 2001) has been shown to affect heat-shock-dependent release of FGF-1 (Carreira et al., 1998; Landriscina et al., 2001). Other proteins secreted in an ER/Golgi-independent manner such as IL1β and the galectins are increasingly exported upon heat shock as well (for a review, see Hughes, 1999); however, FGF-2 export is unaffected by this treatment (Mignatti et al., 1992). Other differences concern the involvement of intracellular vesicles in the overall export process of non-conventionally secreted proteins. Studies have shown that whereas the galectins accumulate underneath the plasma membrane followed by a release mechanism termed membrane blebbing that appears to involve exosomes (Mehul and Hughes, 1997), IL1β is taken up by intracellular vesicles that have been defined as an endo-lysosomal compartment (Andrei et al., 1999) that fuses with the plasma membrane in order to release IL1β. No such data have been reported for either FGF-1 or FGF-2 [for reviews of the various kinds of non-conventional protein secretion, see Cleves, 1997 and Hughes, 1999 (Cleves, 1997; Hughes, 1999)]. In conclusion, it may be that distinct mechanisms exist promoting ER/Golgi-independent export of various secretory proteins from mammalian cells.

In the current study we introduce a novel assay that reconstitutes non-conventional secretion of biosynthetic FGF-2 in living cells. By using stable cell lines and flow cytometry, FGF-2-GFP export can be determined on a quantitative basis. FGF-2-GFP fusion proteins are expressed in a doxicyclin-dependent manner in CHO cells followed by their translocation to the extracellular surface of the plasma membrane. It is shown that both N- and C-terminal GFP-tagging is compatible with this process, which is demonstrated to occur by a controlled mechanism rather than by unspecific release. Based on its design the experimental system described will be useful both for studying the molecular mechanism of FGF-2 secretion and for high throughput screening for inhibitors of this process.

In the second part of this study we made use of the system described in order to analyze the fate of biosynthetic FGF-2 (rather than exogenously added FGF-2) following its translocation to the extracellular compartment. Secreted FGF-2-GFP is shown to accumulate in large HSPG-containing protein clusters that are distinct from caveolae-like lipid rafts. While CHO wild-type cells lack high-affinity FGF receptors (Rusnati et al., 2002), they do express the complex ganglioside GM_1, which has been shown to be required for FGF-2 signalling (Rusnati et al., 2002). Therefore, it can be concluded that FGF receptors are required to target HSPG-bound FGF-2 to caveolae-like lipid rafts in order to initiate FGF-2 signalling (Davy et al., 2000). Consistently, neither endogenous FGF-2-GFP exported to the cell surface nor exogenously added recombinant FGF-2 stimulate CHO cells with regard to cell proliferation. FGF-2 export and FGF-2 deposition in HSPG-containing clusters are not tightly linked processes as we demonstrate a soluble intermediate that is secreted into the culture medium. Moreover, under the conditions applied, the availability of HSPGs on the cell surface is not a rate-limiting step in the overall process of FGF-2 secretion. In conclusion, the experimental system presented in this study reconstitutes the whole pathway of FGF-2 biogenesis in living cells, including both non-conventional secretion and deposition of FGF-2 in HSPG-containing microdomains on the surface of CHO cells. By the use of CHO cells, FGF-2 export does not cause autocrine FGF-2 signalling, which makes this system especially suited to the study of the molecular mechanism of non-conventional FGF-2 secretion.

Materials and Methods

Recombinant proteins and antibodies

A His_6-FGF-2 construct was generated based on a PCR product corresponding to the 18 kDa isoform of FGF-2 and the vector pET15b (Novagen). Similarly, a His_6-eGFP construct was generated. Recombinant His_6-FGF-2 and His_6-eGFP were expressed in *E. coli* BL21(DE3) cells and purified from a 100,000 $\boldsymbol{g}_{av}$ supernatant of homogenized cells by using Ni-NTA agarose (Qiagen) according to standard procedures. Homogenous preparations of His_6-FGF-2 and His_6-eGFP were obtained with protein concentrations of 6 mg/ml and 2.5 mg/ml, respectively.

Anti-GFP antibodies were generated by immunization of rabbits with recombinant N-terminally His_6-tagged eGFP expressed in *E. coli*. The resulting anti-serum was incubated with His_6-tagged eGFP coupled to epoxy sepharose (Amersham Pharmacia). Bound antibodies were eluted under acidic conditions according to standard procedures. In the same way, affinity-purified anti-FGF-2 antibodies were generated using recombinant N-terminally His_6-tagged FGF-2 (18 kDa isoform).

Polyclonal rat anti-CD2 antibodies (LFA-2) as well as polyclonal rabbit anti-caveolin-1 (N-20) antibodies were obtained from Southern Biotechnology Associates and Santa Cruz Biotechnology, respectively. Phycoerythrin-coupled anti-rabbit-IgG were from Molecular Probes. Polyclonal antibodies directed against the cytoplasmic domain of the Golgi transmembrane protein p23 [#1402, (Sohn et al., 1996)] were kindly provided by Felix Wieland (Biochemie-Zentrum Heidelberg).

Generation of model cell lines for the reconstitution of FGF-2 secretion in living cells

CHO wild-type cells (ECACC; Ref. No. 85050302) were genetically modified by the stable integration of cDNAs encoding the murine cation transporter MCAT-1 (Albritton et al., 1989; Davey et al., 1997), the doxicyclin-sensitive transactivator rtTA2-M2 (Urlinger et al., 2000), a truncated version of the cell surface protein CD2 (Liu et al., 2000), and one of three reporter molecules (N-FGF-2-GFP-C, N-GFP-FGF-2-C and N-GFP-C) for each cell line. Throughout this study, the open reading frame of enhanced GFP (eGFP, Clontech) has been used

for the generation of all cDNA constructs. In the first step the open reading frame of MCAT-1 was subcloned into the vector pcDNA3zeo$^+$ (Invitrogen), which carries the zeocin-resistance gene. Following transfection of CHO cells with pcDNA3zeo$^+$-MCAT DNA, cells were selected with medium containing 400 μg/ml zeozin. After about 14 days individual clones were pooled to generate a heterogenous population of zeozin-resistant CHO cells termed CHO_{MCAT}. CHO_{MCAT} cells were then transduced with retroviral particles produced from HEK-293T cells by co-transfection of the vectors pVPack-GP and pVPack-eco (Stratagene) as well as pBI-CD2 (Liu et al., 2000) carrying a bicistronic construct encoding the Doxicyclin-sensitive transactivator rtTA2-M2 and a truncated version of CD2 under the control of a constitutive promotor based on the vector. After three days, 50,000 CD2-positive cells were isolated by FACS sorting using polyclonal anti-CD2-antibodies detected by anti-rat secondary antibodies coupled to Alexa488 (Dianova). This pool of CHO cells was termed CHO_{TAM2}. Following 7 days of incubation at 37°C, the corresponding population of cells was transduced with retroviral particles carrying one of the three reporter molecules mentioned above. In this case, the cDNA constructs (N-FGF-2-eGFP-C, N-eGFP-FGF-2-C and N-eGFP-C) were subcloned into the vector pREV-TRE2 (Clontech), which contains a Doxicyclin/transactivator-responsive element for the initiation of mRNA formation. The open reading frames of FGF-2 and eGFP originated from the vector pT7T3D-Pac (FGF-2, IMAGE Consortium no. 1690025) and the vector pEGFP1 (eGFP, Clontech). Three days after retroviral transduction, including 12 hours of incubation in the presence of 1 μg/ml doxicyclin (Sigma), 50,000 cells from each transduction sample were isolated by FACS sorting based on GFP-derived fluorescence. The three pools of cells were incubated for 7 days at 37°C in the absence of doxicyclin followed by the isolation of 50,000 cells from each population that did not display GFP-derived fluorescence at this point. Each population was now cultured for another 7 days at 37°C including 12 hours in the presence of 1 μg/ml doxicyclin at the end of this procedure. Single cells were isolated by FACS sorting based on GFP-derived fluorescence. These clones were propagated and used for the preparation of frozen stocks. The newly generated clonal cell lines were termed $CHO_{FGF\text{-}2\text{-}GFP}$, $CHO_{GFP\text{-}FGF\text{-}2}$ and CHO_{GFP}, respectively, in order to reflect the reporter molecule expressed.

Biochemical analysis of FGF-2-GFP secretion

$CHO_{FGF\text{-}2\text{-}GFP}$, $CHO_{GFP\text{-}FGF\text{-}2}$ and CHO_{GFP} cells were grown on 6-well plates for 36 hours at 37°C in the presence of 1 μg/ml doxicyclin and 125 μg/ml heparin. Where indicated the cells were incubated in the presence of 25 μM ouabain (Sigma). The medium was removed followed by the dissociation of the cells from the culture plates using a protease-free buffer (Gibco, PBS-based cell dissociation buffer) supplemented with 125 μg/ml heparin. Following sedimentation of cells, the supernatant was combined with the original medium, diluted 1:10 in a Tris buffer (10 mM, pH 7.4) containing 1 mM EDTA and 1% (w/v) Triton X-100. The cells were lysed in the same buffer. Both the cellular extracts and the corresponding supernatants were then subjected to FGF-2 affinity purification using heparin sepharose (Amersham Pharmacia). Bound material was eluted with SDS sample buffer followed by SDS-PAGE and western blot analysis using affinity-purified anti-GFP antibodies and ECL detection (Amersham Pharmacia). In the case of CHO_{GFP} cells, aliquots from both the cellular and the medium fractions were combined with sample buffer followed by SDS-PAGE and western blot analysis in order to determine the distribution of the reporter molecule (GFP) between cells and medium. Since the medium contains about 5-10 μg/μl total protein (derived from the fetal calf serum), it was not possible to apply more than 1% of the medium (10 μl out of 1000 μl per sample) to the gel. However, even after prolonged exposure GFP could not be detected in the medium fraction.

Confocal microscopy

$CHO_{FGF\text{-}2\text{-}GFP}$ and CHO_{GFP} cells were grown on glass coverslips for 36 hours at 37°C in the presence of 1 μg/ml doxicyclin. The cells were then processed, including paraformaldehyde fixation (3% w/v, 20 minutes at 4°C) without permeabilization, followed by antibody processing as indicated. Alexa546-coupled secondary antibodies (Dianova) were used for cell surface staining experiments. The specimens were mounted in Fluoromount G (Southern Biotechnology Associates) and viewed with a Zeiss LSM 510 confocal microscope.

Fluorescence activated cell sorting

$CHO_{FGF\text{-}2\text{-}GFP}$, $CHO_{GFP\text{-}FGF\text{-}2}$ and CHO_{GFP} cells were grown under the conditions indicated in the corresponding figure legends. To detach the cells from the culture plates without using protease-based protocols, cell dissociation buffer (Life Technologies) was used to generate a cell suspension devoid of cell aggregates. Where indicated, cells were treated with antibodies for 1 hour at 4°C on a rotating wheel. Wash procedures were carried out by sedimenting the cells at 200 ***g*** for 5 minutes at 4°C. Prior to the FACS analysis, propidium iodide (1 μg/ml) was added in order to detect damaged cells.

GFP- and phycoerythrin-derived fluorescence (Molecular Probes) was analyzed using a Becton Dickinson FACScan flow cytometer. Autofluorescence was determined by measuring non-induced cells that were not treated with phycoerythrin-coupled secondary antibodies. GFP-positive cells (i.e. grown in the presence of 1 μg/ml doxicyclin) that were not treated with antibodies were used to appropriately compensate the FL-2 channel used to detect phycoerythrin-derived fluorescence.

Isolation of detergent-insoluble microdomains

$CHO_{FGF\text{-}2\text{-}GFP}$ cells were grown on large culture plates (15 cm diameter) for 36 hours in the presence of doxicyclin (1 μg/ml). The cells were washed twice with PBS followed by the addition of PBS supplemented with 10% (w/v) sucrose. After dissociation from the culture plates using a rubber policeman, cell disruption was achieved using a Balch homogenizer (Balch and Rothman, 1985). The resulting suspension was subjected to differential centrifugation at 1000 ***g*** and 5000 ***g***, respectively. The 5000 ***g*** supernatant was loaded onto a 20% sucrose cushion followed by ultracentrifugation at 100,000 ***g*** for 60 minutes at 4°C. The resulting membrane sediment represents a microsomal membrane fraction containing intracellular as well as plasma membranes. The preparation of detergent-soluble and -insoluble fractions as well as the flotation analysis using sucrose gradients were performed as described (Gkantiragas et al., 2001).

Results

Generation of model cell lines to study non-classical export of FGF-2

CHO cells were chosen to establish an experimental system that allows reconstitution of non-classical FGF-2 secretion in vivo. The generation of these cell lines was achieved in three steps (see Materials and Methods). First, the murine orthologue of the cationic amino acid transporter MCAT-1 (Albritton et al., 1989; Davey et al., 1997) was stably transfected into CHO cells based on the vector pcDNA3-zeo, which contains the zeocin-resistance gene. Cell surface expression of MCAT-1 renders CHO cells permissive for retroviral transduction based on the ecotropic surface protein of murine leukemia virus (Albritton et al., 1989; Davey et al., 1997). Accordingly, a pool of zeocin-resistant cells could be transduced with an ecotropic retrovirus carrying a bicistronic construct consisting of the doxicyclin-sensitive transactivator rtTA2-M2 (Urlinger et al.,

2000) and a truncated version of CD2 (Liu et al., 2000) that was used as a cell surface marker. A pool of CD2-positive cells was isolated by FACS sorting and subjected to another round of retroviral transduction. This time a retroviral vector carrying a doxicyclin/transactivator-dependent promotor was used to generate three different kinds of cell lines: one expressing N-terminally GFP-tagged FGF-2, one expressing C-terminally GFP-tagged FGF-2 and one expressing GFP alone. In each case, a pool of positive cells was selected by GFP-derived fluorescence following incubation in the presence of doxicyclin. After repeated FACS sorting in the absence and presence of doxicyclin (for details, see Materials and Methods), single cells were isolated that were found to be positive for MCAT-1, rtTA2-M2 and CD2 as well as FGF-2-GFP, GFP-FGF-2 or GFP, respectively. These cells were designated $CHO_{FGF\text{-}2\text{-}GFP}$, $CHO_{GFP\text{-}FGF\text{-}2}$ and CHO_{GFP} in order to reflect the reporter molecule expressed.

The characterization of CHO clones derived from the procedure described above is shown in Fig. 1 (see Materials and Methods). As depicted in Fig. 1A, a PCR analysis of genomic DNA revealed the presence of DNA fragments of the expected size (lanes 1-3) compared with the vector DNA (lanes 4-6) that was used for retroviral transduction. In Fig. 1B, total extracts from each clone were separated on SDS gels followed by western blotting and immunodetection using affinity-purified anti-GFP antibodies. For each clone, an immunoreactive band with an apparent migration behaviour corresponding to about 45 (FGF-2-GFP, GFP-FGF-2) and 26 (GFP) kDa, respectively, was observed when cells were incubated in the presence of doxicyclin (Fig. 1B, lanes 2,4,6). By contrast, no signal was observed when cells were incubated in the absence of doxicyclin (Fig. 1B, lanes 1,3,5). These results were confirmed by fluorescence microscopy (Fig. 1C-H) and flow cytometry (Fig. 1I-K). Upon incubation of each CHO clone in the presence of doxicyclin, the whole cell population displayed increased GFP fluorescence (~50-100-fold), as determined by flow cytometry. Based on conventional fluorescence microscopy at low magnification, FGF-2-GFP and GFP-FGF-2 display both nuclear and cytoplasmic staining. This is also the case for GFP; however, the ratio of nuclear to cytoplasmic staining is significantly lower compared with that of FGF-2-GFP and GFP-FGF-2.

Biochemical analysis of FGF-2 fusion protein secretion

To functionally characterize the reporter cell lines with regard to non-classical secretion, we conducted biochemical experiments to assess extracellular localization of biosynthetic FGF-2-GFP fusion proteins (see Materials and Methods). For this purpose, cells were exposed to doxicyclin for 48 hours at 37°C in the presence of heparin (to prevent FGF-2 binding to plasma-membrane-associated HSPGs), followed by their dissociation from the culture plates by using a protease-free protocol. Residual cell surface-associated FGF-2 was released by heparin and the corresponding cell-free supernatant was combined with the original growth medium. In parallel, detergent extracts from the cellular fractions were

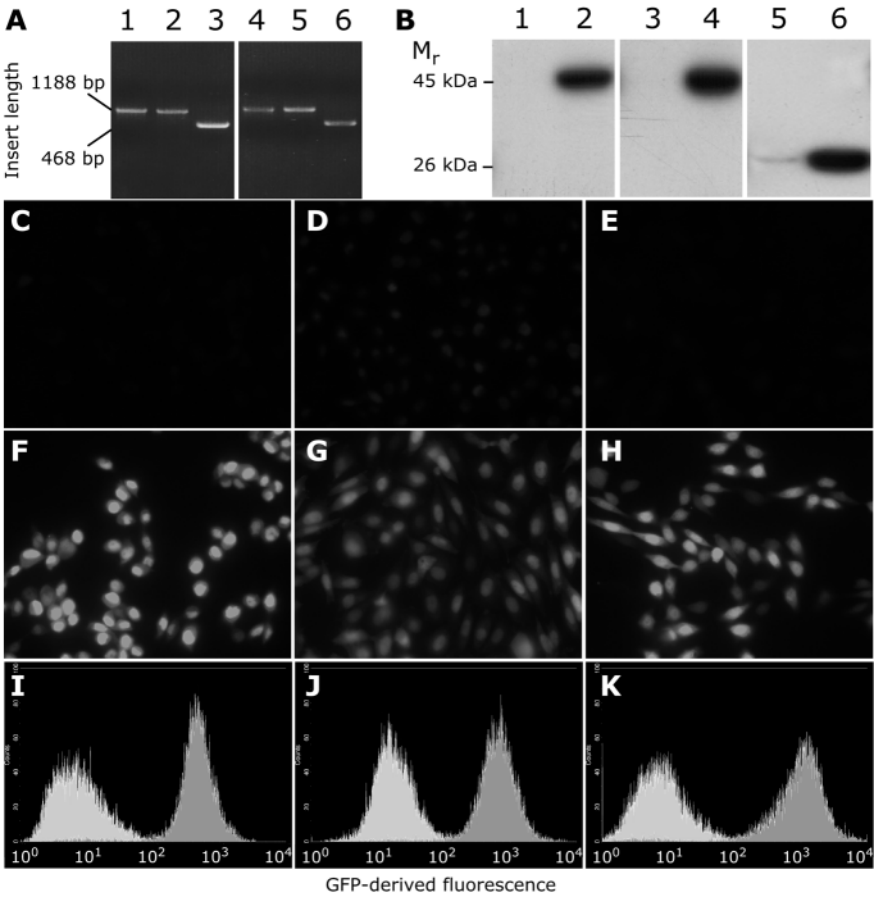

Fig. 1. Characterization of the cell lines $CHO_{FGF\text{-}2\text{-}GFP}$, $CHO_{GFP\text{-}FGF\text{-}2}$ and CHO_{GFP}. The model cell lines generated to study non-conventional export of FGF-2 were characterized with regard to genomic cDNA integration (A), western blot analysis of doxicyclin-dependent protein expression (B), analysis of doxicyclin-dependent protein expression based on fluorescence microscopy (C-H), analysis of doxicyclin-dependent protein expression based on FACS (I-K). (A) PCR analysis: $CHO_{FGF\text{-}2\text{-}GFP}$ (lanes 1,4); $CHO_{GFP\text{-}FGF\text{-}2}$ (lanes 2,5); CHO_{GFP} (lanes 3,6). Lanes 1-3 represent PCR reactions using genomic DNA as template isolated from the cell lines indicated; lanes 4-6 represent PCR reactions using the original retroviral plasmids as template (positive controls). (B) Western blot analysis: $CHO_{FGF\text{-}2\text{-}GFP}$ (lanes 1,2); $CHO_{GFP\text{-}FGF\text{-}2}$ (lanes 3,4); CHO_{GFP} (lanes 5,6). Total cell lysates (20 μg protein/lane) were subjected to SDS-PAGE followed by a western blot analysis using affinity-purified anti-GFP antibodies. Lanes 1, 3 and 5 correspond to cell cultures incubated in the absence of doxicyclin; lanes 2, 4 and 6 correspond to cultures incubated in the presence of doxicyclin. (C-H) Fluorescence microscopy analysis: $CHO_{FGF\text{-}2\text{-}GFP}$ (C,F); $CHO_{GFP\text{-}FGF\text{-}2}$ (D,G); CHO_{GFP} (E,H). Panels C, D and E represent cell cultures incubated in the absence of doxicyclin; panels F, G and H represent cell cultures incubated in the presence of doxicyclin. (I-K) FACS analysis: $CHO_{FGF\text{-}2\text{-}GFP}$ (I); $CHO_{GFP\text{-}FGF\text{-}2}$ (J); CHO_{GFP} (K). The cell populations grown in the absence of doxicyclin are shown in white, those grown in the presence of doxicyclin are shown in grey.

prepared. Following dilution, FGF-2 fusion proteins were affinity-purified from both the cellular and the medium fractions using heparin sepharose (Klagsbrun et al., 1987). As depicted in Fig. 2A, both FGF-2-GFP (derived from the cell line $CHO_{FGF\text{-}2\text{-}GFP}$) and GFP-FGF-2 (derived from the cell line $CHO_{GFP\text{-}FGF\text{-}2}$) were readily detectable in the supernatant of cultured cells (lanes 2 and 4, respectively). Since 1% of the total material derived from cells (lanes 1 and 3, respectively) and 15% of the total material derived from the cell supernatant (lanes 2 and 4, respectively) were applied to the gel, up to 10% of the FGF-2-GFP fusion proteins expressed were found to be secreted under the experimental conditions applied. To analyze the specificity of FGF-2 export, the corresponding control cell line (CHO_{GFP}) was used, which expressed GFP without being fused to FGF-2. As can be deduced from lanes 5 (cells) and 6 (supernatant) of Fig. 2A, GFP could not be detected in the supernatant of CHO_{GFP} cells. As depicted in Fig. 2B, $CHO_{FGF\text{-}2\text{-}GFP}$ cells that were grown in the presence of ouabain, a known inhibitor of FGF-2 secretion (Florkiewicz et al., 1998; Dahl et al., 2000), FGF-2-GFP export to the culture medium was markedly reduced [compare the ratio of the relative amounts of FGF-2-GFP in lanes 1 and 2 (control) to the corresponding ratio of lanes 3 and 4 (ouabain)]. These data establish that export of FGF-2-GFP fusion proteins from CHO cells is a specific transport mechanism that is compatible with both N- and C-terminal GFP tagging.

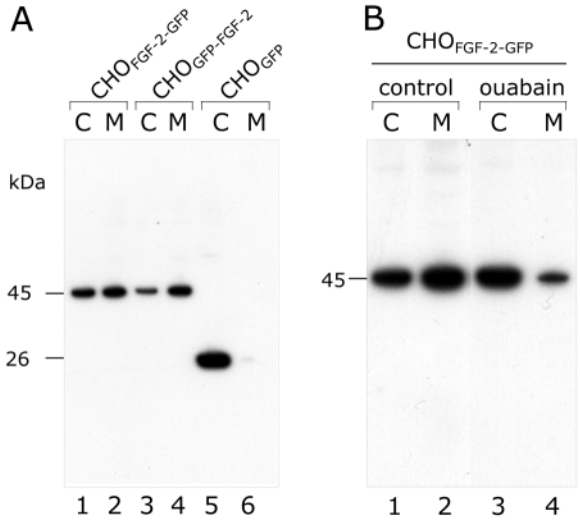

Fig. 2. Biochemical analysis of FGF-2 fusion protein secretion. The various cell lines indicated were analyzed biochemically with regard to secretion of the reporter molecules (A). Cells were grown in the presence of doxicyclin and heparin for 48 hours at 37°C. FGF-2-GFP and GFP-FGF-2 were affinity-purified from detergent cell extracts and the medium by using heparin sepharose. 1% (cells) and 15% (medium) of the eluates were subjected to SDS-PAGE. In case of CHO_{GFP} cells, 1% of both cells and medium were directly subjected to SDS-PAGE (the amount of the medium loaded onto the gel had to be reduced to 1% of the total material because of the high protein concentration). Affinity-purified anti-GFP antibodies were used to detect the reporter molecules. Even after prolonged exposition, no GFP signal could be observed in lane 6. To analyze whether FGF-2-GFP is released by a specific mechanism, $CHO_{FGF\text{-}2\text{-}GFP}$ cells were grown for 48 hours at 37°C in the presence of doxicyclin, 125 μg/ml heparin and 25 μM ouabain, a drug known to inhibit FGF-2 export (B). The samples were processed as described above.

Determination of FGF-2 translocation to the cell surface based on flow cytometry

To analyze FGF-2 export to the cell surface in living cells, we have established a novel assay that is based on flow cytometry (see Materials and Methods). $CHO_{FGF\text{-}2\text{-}GFP}$ cells were incubated for 18 hours at 37°C in the presence of doxicyclin. Following dissociation from the culture plates using a protease-free buffer, cells were incubated at 4°C under native conditions with affinity purified antibodies directed against either GFP or FGF-2. Primary antibodies were detected by secondary antibodies coupled to phycoerythrin (PE) in order to visualize cell surface localization by PE-derived fluorescence. As depicted in Fig. 3, GFP-derived fluorescence rigorously depends on the presence of doxicyclin as shown by dot blots (compare Fig. 3A and 3B) as well as the corresponding histogram (Fig. 3F). Under all experimental conditions, the degree of doxicyclin-dependent GFP fluorescence was found to be similar (Fig. 3F). Accordingly, PE-derived fluorescence corresponding to cell-surface-localized FGF-2-GFP could only be observed when FGF-2-GFP expression was induced by doxicyclin, which demonstrated the monospecificity of the affinity-purified anti-GFP antibodies used. Similar results were obtained with affinity-purified anti-FGF-2 antibodies (data not shown).

To further establish that PE-derived fluorescence exclusively represented cell surface localization of FGF-2-GFP, we conducted experiments where native cells were treated with trypsin prior to the FACS analysis (Fig. 3E,G). In addition, experiments were carried out where cells were incubated with heparin in order to elute FGF-2-GFP associated with plasma-membrane-bound heparan sulfate proteoglycans (Fig. 3D,G). In both cases, the majority of PE-derived fluorescence could be removed from the cells demonstrating that the signal was derived from a FGF-2-GFP population associated with the outer surface of the plasma membrane.

FGF-2 translocation to the cell surface depends on the FGF-2 domain of FGF-2-GFP fusion proteins

To analyze whether the FGF-2 domain is required for the translocation to the cell surface of FGF-2-GFP fusion proteins, we compared the various CHO cell lines described in Fig. 1 with regard to their ability to translocate the respective reporter molecule to the outer surface of the plasma membrane. The three CHO clones were incubated in the presence of doxicyclin for 18 hours (see Materials and Methods) followed by antibody processing as described in the legend of Fig. 3. As shown in Fig. 4A, total GFP-derived fluorescence differed in the three cell lines. When the autofluorescence-corrected GFP signal of $CHO_{FGF\text{-}2\text{-}GFP}$ cells was set to 100%, $CHO_{GFP\text{-}FGF\text{-}2}$ and CHO_{GFP} cells displayed a 1.4-fold and 2.3-fold higher fluorescence, respectively, compared with that of $CHO_{FGF\text{-}2\text{-}GFP}$ cells. By contrast, cell surface localization of the respective reporter molecules as measured by PE-derived fluorescence (Fig. 4B) was observed only with $CHO_{FGF\text{-}2\text{-}GFP}$ and $CHO_{GFP\text{-}FGF\text{-}2}$ cells. Again, the autofluorescence-corrected signal of $CHO_{FGF\text{-}2\text{-}GFP}$ cells was set to 100% and shown to be 14-fold higher compared with CHO_{GFP} cells. The cell surface signal of $CHO_{GFP\text{-}FGF\text{-}2}$ was slightly higher than that of $CHO_{FGF\text{-}2\text{-}GFP}$ cells; however, the expression level between these two cell lines also differed to a similar extent. These data establish that

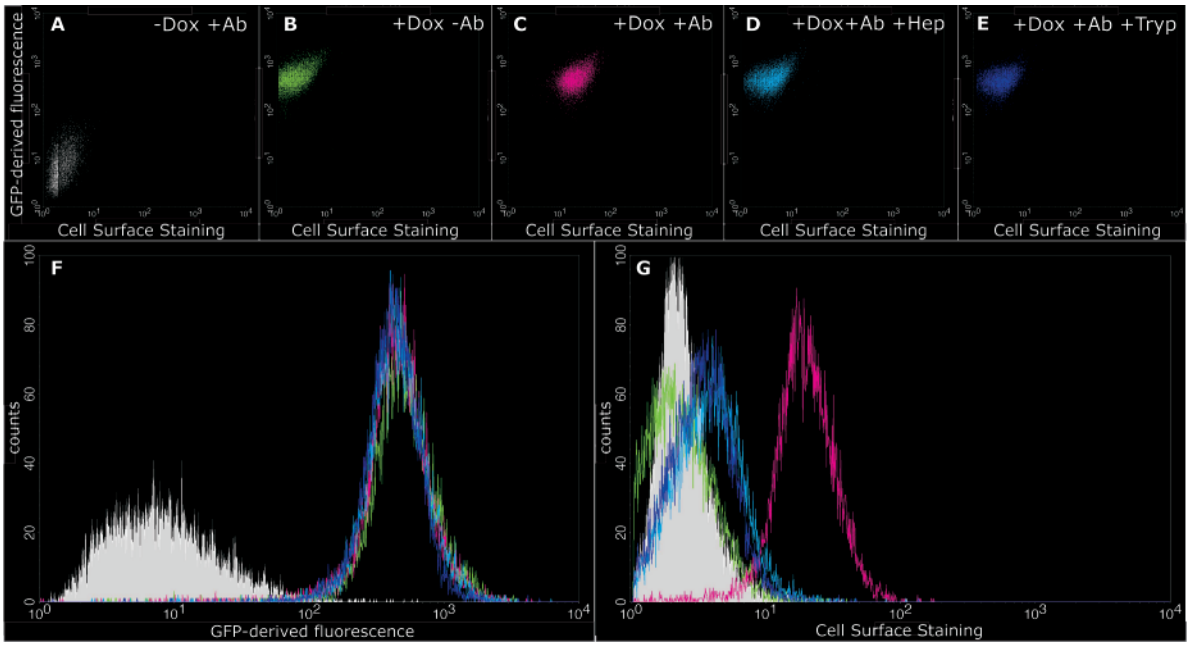

Fig. 3. A novel experimental system to quantitatively determine FGF-2 export from living CHO cells. $CHO_{FGF\text{-}2\text{-}GFP}$ cells were grown for 18 hours at 37°C under the conditions indicated followed by dissociation from the culture plates by using a protease-free protocol. The cell suspension was then processed for the FACS analysis under the conditions indicated. Panels A-E represent dot blots where total GFP-derived fluorescence was blotted against cell surface-derived PE fluorescence. (A) Cells grown in the absence of doxicyclin. (B) Cells grown in the presence of doxicyclin and processed with anti-GFP antibodies. (C) Cells grown in the presence of doxicyclin and processed with anti-GFP antibodies. (D) Cells grown in the presence of doxicyclin followed by a wash procedure using a heparin-containing buffer and antibody processing. (E) Cells grown in the presence of doxicyclin followed by trypsin digestion and antibody processing. Panels F and G represent the corresponding histograms of GFP-derived fluorescence and PE-derived cell surface fluorescence, respectively. The colours correspond to the conditions shown in panels A-E.

the translocation process depends on the FGF-2 domain of the reporter molecules, which is consistent with the biochemical data presented in Fig. 2. Moreover, it is shown that both N-terminal and C-terminal GFP-tagging of FGF-2 is compatible with the translocation process in CHO cells.

Characterization of FGF-2 translocation to the cell surface

In a series of experiments depicted in Fig. 5, we characterized the FACS-based FGF-2 translocation assay with regard to kinetics, unspecific release as well as sensitivity to ouabain, a known inhibitor of FGF-2 export (Florkiewicz et al., 1998; Dahl et al., 2000). Following induction of protein expression in the presence of doxicyclin, the amount of FGF-2 appearing on the cell surface increased in a linear manner for up to 48 hours (Fig. 5A). Afterwards, the signal turned into saturation, indicating that steady-state conditions were reached. To rule out that the material found on the cell surface was derived from damaged cells, experiments were carried out where $CHO_{FGF\text{-}2\text{-}GFP}$ cells cultured in the absence of doxicyclin were incubated with various amounts of a supernatant derived from

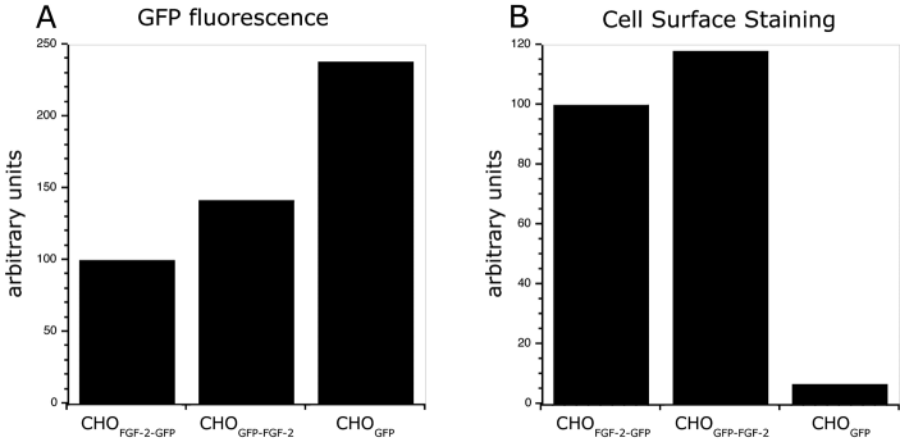

Fig. 4. Translocation to the cell surface of FGF-2 fusion proteins depends on the FGF-2 domain and is compatible with both N- and C-terminal GFP tagging. $CHO_{FGF\text{-}2\text{-}GFP}$, $CHO_{GFP\text{-}FGF\text{-}2}$ and CHO_{GFP} cells were grown in the presence of doxicyclin for 18 hours at 37°C followed by FACS processing, including antibody treatment, as described in Materials and Methods. (A) Quantitative comparison of GFP-derived fluorescence. (B) Quantitative comparison of PE-derived cell surface fluorescence. For both GFP- and PE-derived fluorescence, the signal produced by $CHO_{FGF\text{-}2\text{-}GFP}$ cells was set to 100. The results shown are representative of two independent experiments.

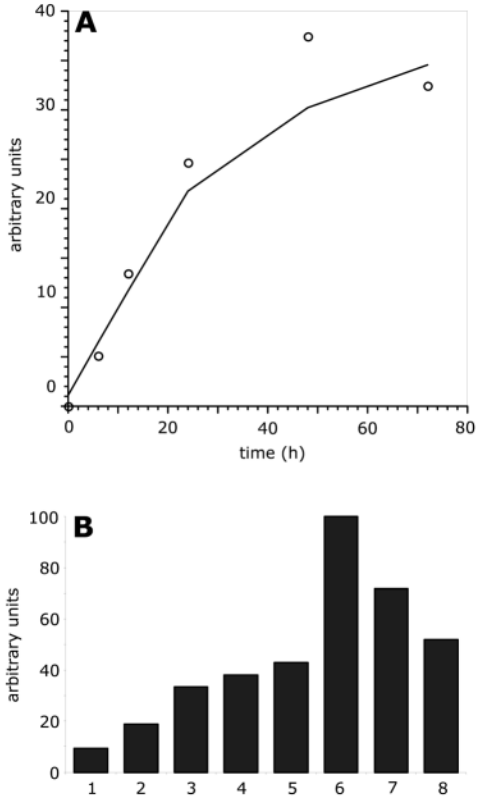

Fig. 5. Characterization of FGF-2-GFP export with regard to kinetics, unspecific release and inhibition by ouabain. (A) Kinetic analysis of FGF-2-GFP export. $CHO_{FGF\text{-}2\text{-}GFP}$ cells were grown in the presence of doxicyclin for the times indicated followed by FACS processing, including antibody treatment, as described in Materials and Methods. The raw data have been subjected to a weighted curve fit and are representative of two independent experiments.
(B) $CHO_{FGF\text{-}2\text{-}GFP}$ cells were grown in the absence of doxicyclin followed by the addition of various amounts of a supernatant derived from homogenized $CHO_{FGF\text{-}2\text{-}GFP}$ cells that were grown for 48 hours in the presence of doxicyclin (lanes 1-5). Based on cell number, 0% (lane 1), 2.5% (lane 2), 5% (lane 3), 7.5% (lane 4) and 10% (lane 5) of this supernatant was added to $CHO_{FGF\text{-}2\text{-}GFP}$ cells grown in the absence of doxicyclin. The PE-derived FGF-2-GFP cell surface signal was then compared with the corresponding signal of $CHO_{FGF\text{-}2\text{-}GFP}$ cells grown for 48 hours in the presence of doxicyclin (set to 100%, lane 6). Lanes 7 and 8 refer to experiments under the same conditions as those in lane 6 with the exception that during the whole course of the experiment, 1 μM and 5 μM ouabain, respectively, were added to the culture medium. The data are representative of two independent experiments.

homogenized $CHO_{FGF\text{-}2\text{-}GFP}$ cells cultured in the presence of doxicyclin for 48 hours at 37°C (see Materials and Methods). As shown in Fig. 5B, the addition of 0, 2.5, 5, 7.5 or 10% (based on cell number; lanes 1-5) of a supernatant derived from a membrane-free supernatant of homogenized cells to cells not expressing the reporter molecule accounted for up to 40% of the secretion signal observed with $CHO_{FGF\text{-}2\text{-}GFP}$ cells (lane 6). During all FACS experiments the amount of dead cells was monitored by the addition of propidium iodide (PI), a low molecular weight dye that enters only damaged cells. Typically, about 2-3% of the total cell population was found to be positive for PI. Thus, the population of FGF-2-GFP found on the cell surface (lane 6) cannot be derived from damaged cells but rather has been secreted by a specific transport mechanism. This conclusion is further substantiated by the observation that the appearance of FGF-2-GFP on the cell surface can be partially inhibited by ouabain (Fig. 5B, lanes 7,8). The results obtained by this FACS analysis are consistent with the biochemical secretion experiments shown in Fig. 2.

The amount of HSPGs on the cell surface does not limit FGF-2 translocation efficiency

To analyze the influence of HSPG levels on the cell surface with regards to FGF-2 secretion efficiency we conducted experiments where recombinant His_6-FGF-2 (see Materials and Methods) was titrated into the medium of both doxicyclin-induced and non-induced $CHO_{FGF\text{-}2\text{-}GFP}$ cells. As depicted in Fig. 6, the FGF-2 binding capacity of the cells was not saturated under conditions where FGF-2-GFP expression and externalization was induced by doxicyclin. Based on these results, the binding capacity of $CHO_{FGF\text{-}2\text{-}GFP}$ cells for FGF-2 is at least ten times higher than the amount of FGF-2-GFP externalized under the conditions described. Therefore, the FGF-2 secretion signal observed is not limited by the amount of HSPGs available on the cell surface but rather is a precise measure of the efficiency of the export machinery. Thus, it can be concluded that the overall process of FGF-2 externalization is not governed by a balance of intracellular FGF-2 versus extracellular HSPG-bound FGF-2. Rather, the FGF-2 export machinery appears to be rate-limiting under the conditions applied.

Analysis of FGF-2 translocation to the cell surface by confocal microscopy

To verify the results obtained by FACS analysis using an independent method, we conducted experiments based on immunofluorescence confocal microscopy (see Materials and Methods). $CHO_{FGF\text{-}2\text{-}GFP}$ and CHO_{GFP} cells were grown on glass coverslips for 24 hours in the absence or presence of doxicyclin. Following fixation (without permeabilization) and antibody processing using affinity-purified anti-GFP antibodies, the various samples were analyzed by confocal microscopy. As shown in Fig. 7B,F, $CHO_{FGF\text{-}2\text{-}GFP}$ cells incubated in the presence of doxicyclin displayed both GFP-derived intracellular fluorescence (both the nucleus and the cytoplasm were found to be positive for FGF-2-GFP) and plasma-membrane-associated Alexa546-derived fluorescence. By contrast, $CHO_{FGF\text{-}2\text{-}GFP}$ cells incubated in the absence of doxicyclin (Fig. 7A,E) neither showed GFP-derived fluorescence nor cell surface staining, demonstrating monospecificity of the antibodies used. Consistent with the FACS experiments shown in Fig. 3, FGF-2-GFP cell surface staining can be removed by incubation of the cells with heparin prior to fixation (Fig. 7C,G), demonstrating that exported biosynthetic FGF-2-GFP associates with HSPGs on the extracellular surface of CHO cells. Moreover, cell surface staining could not be observed with CHO_{GFP} cells incubated in the presence of doxicyclin (Fig. 7D,H). These results are fully consistent with our FACS analysis establishing specific translocation from the cytosol to the outer surface of the plasma membrane of FGF-2-GFP.

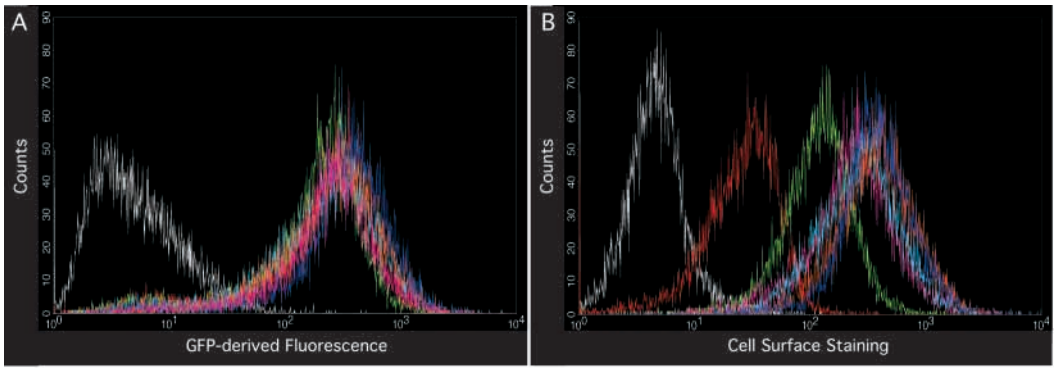

Fig. 6. FGF-2 externalization is not limited by the availability of cell surface HSPGs. $CHO_{FGF\text{-}2\text{-}GFP}$ cells were grown for 18 hours at 37°C in the presence (coloured curves in A and B) or absence (white curves in A and B) of doxicyclin. At the end of the incubation, various amounts of recombinant His_6-tagged FGF-2 were added to the culture medium (green, 0.1 μg/ml; pink, 0.25 μg/ml; blue, 0.5 μg/ml; orange, 1 μg/ml; dark blue, 2 μg/ml) followed by an incubation for 60 minutes at 37°C. The red curves in A and B represent a standard FGF-2 secretion experiment in the absence of exogenously added FGF-2. The cell suspension was processed for the FACS analysis as described in the legend to Fig. 3. In this experiment, affinity-purified anti-FGF-2 antibodies were used to detect both cell surface FGF-2 and FGF-2-GFP. (A) GFP-derived fluorescence. (B) FGF-2 cell surface staining.

Biosynthetic FGF-2-GFP exported to the extracellular plasma membrane surface is targeted to non-lipid raft microdomains

To assess the structural organization of cell-surface-localized FGF-2-GFP in more detail, we conducted immunofluorescence confocal microscopy at high magnification. As shown in Fig. 8A (merged image of 16 confocal planes), FGF-2-GFP did not display a homogenous staining of the plasma membrane but rather appeared in bright spots representing distinct microdomains. As shown in Fig. 8B, these microdomains represent structures exclusively localized to the cell surface since sequential scanning of focal planes (one of which is shown in Fig. 8B) revealed the absence of any intracellular staining. Since cell-surface-associated FGF-2-GFP could be eluted with heparin (Fig. 7G), these microdomains also contain HSPGs.

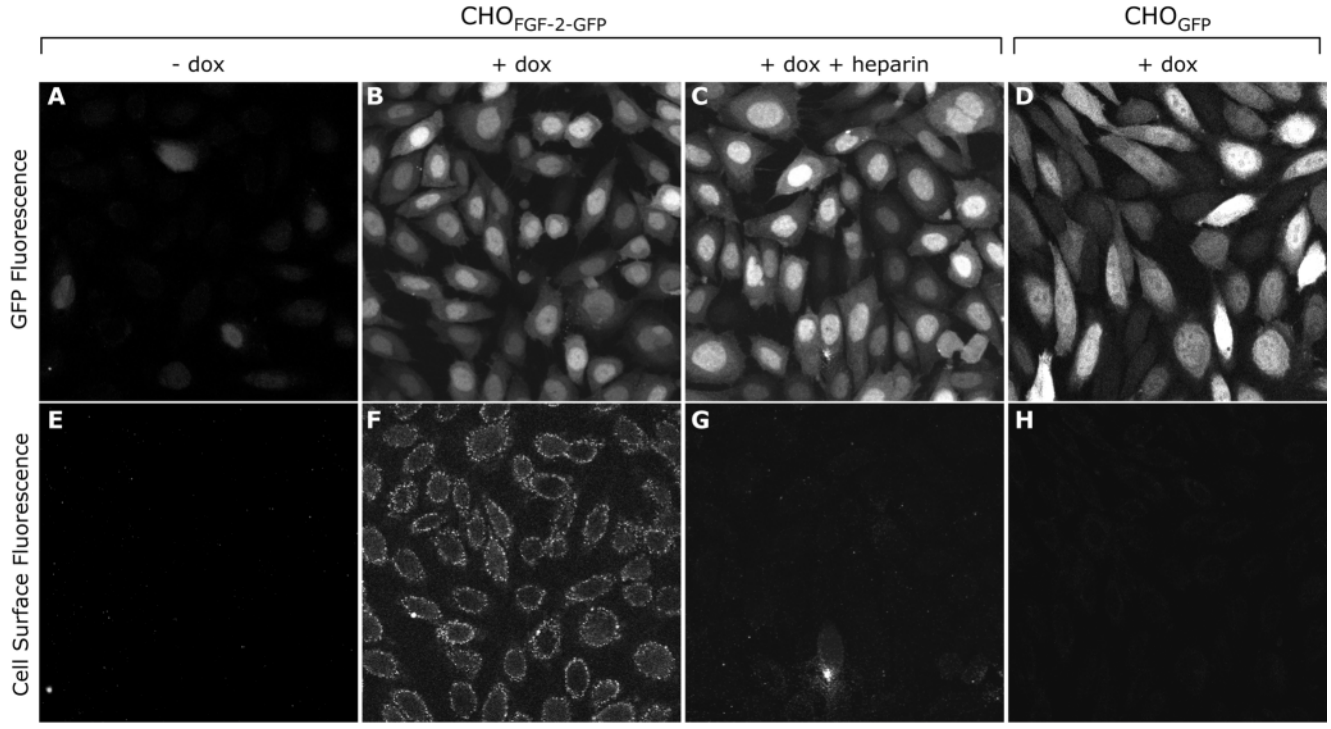

Fig. 7. Translocation of FGF-2-GFP to the extracellular surface of the plasma membrane, as determined by confocal microscopy. $CHO_{FGF\text{-}2\text{-}GFP}$ and CHO_{GFP} cells were grown on glass coverslips for 24 hours at 37°C in the absence or presence of doxicyclin. Where indicated, cells were washed with PBS containing 125 μg/ml heparin. Following fixation using paraformaldehyde, cells were processed with affinity-purified anti-GFP antibodies and secondary antibodies coupled to an Alexa546 fluorophore. The specimens were embedded using Fluoromount and viewed with a Zeiss LSM 510 confocal microscope. The results shown are representative of four independent experiments.

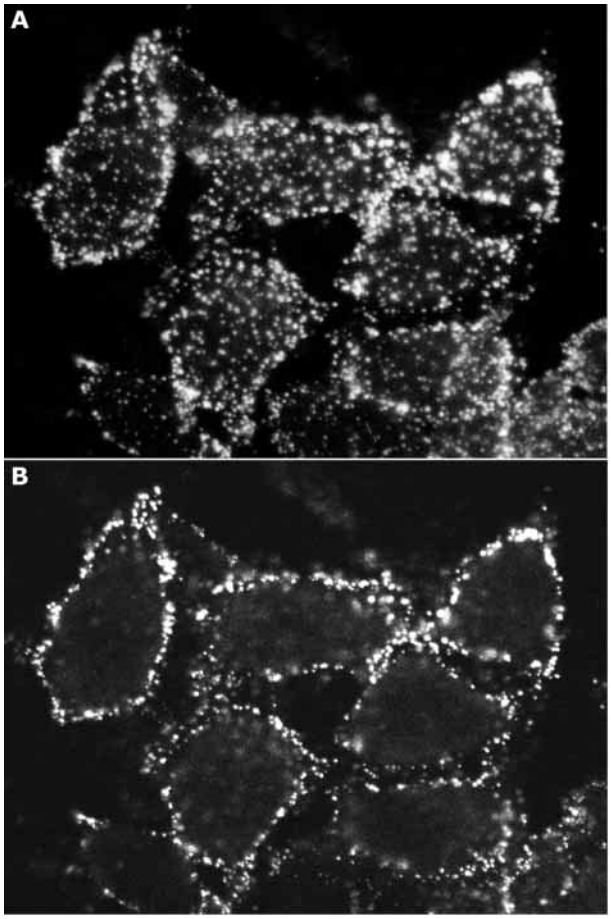

Fig. 8. Identification of FGF-2-GFP-positive microdomains on the extracellular surface of CHO cells. $CHO_{FGF\text{-}2\text{-}GFP}$ cells were grown on glass coverslips for 24 hours at 37°C in the presence of doxicyclin. Processing for confocal microscopy was performed as described in the legend of Fig. 6. (A) Merged image of 16 confocal planes spanning the whole depth of the cells. (B) A confocal plane close to the bottom of the cells where they are attached to the glass coverslips.

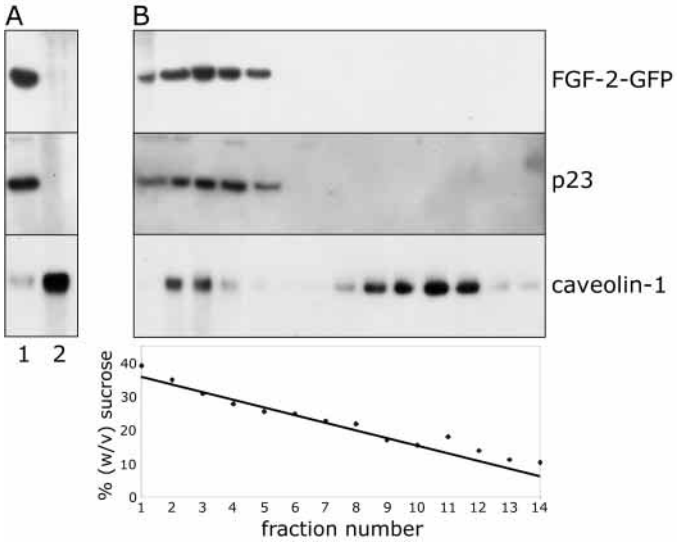

Fig. 9. FGF-2-GFP-positive microdomains are distinct from lipid rafts. $CHO_{FGF\text{-}2\text{-}GFP}$ cells were grown on large culture plates for 48 hours at 37°C in the presence of doxicyclin. Following a wash procedure using PBS the cells were scraped off the culture plates in a sucrose-containing buffer. Cell breakage was achieved by using a balch homogenizer followed by differential centrifugation at 1000 ***g*** and 5000 ***g*** to sediment nuclei and cell debris. The resulting supernatant was loaded on top of a 20% (w/v) sucrose cushion and centrifuged for 60 minutes at 100,000 ***g*** in order to collect microsomal membranes freed of cytosolic proteins. The membrane sediment was resuspended in PEN buffer containing 1% (w/v) Triton X-100 at 4°C. While being resuspended several times using a 100 μl tip, the membrane suspension was kept on ice for 30 minutes. The samples were then divided and either subjected to ultracentrifugation in order to sediment detergent-insoluble complexes or adjusted to 40% (w/v) sucrose followed by flotation in a linear sucrose gradient. (A) Detergent-soluble fraction (lane 1), detergent-insoluble fraction (lane 2). (B) 14 fractions of the linear flotation gradient (lanes 1-14) with lane 1 containing the most dense sucrose fraction and lane 14 containing the lightest fraction. In the case of FGF-2-GFP and p23 detection, 60% of each fraction was TCA-precipitated and applied to the gel; in the case of caveolin-1, 15% of each fraction was TCA-precipitated and applied to the gel.

To analyze the nature of these microdomains with regard to lipid rafts, we characterized the detergent solubility of plasma membrane-associated FGF-2-GFP. A plasma membrane-containing microsomal membrane fraction freed of cell debris, nuclei and soluble cytosolic proteins was isolated by differential centrifugation (see Materials and Methods). These membranes were solubilized in a Triton X-100-containing buffer at 4°C. Resuspended membranes were either subjected to ultracentrifugation in order to sediment detergent-insoluble complexes or adjusted to 40% (w/v) sucrose followed by flotation in a sucrose density gradient in order to separate lipid-associated protein complexes from detergent-soluble material. As shown in Fig. 9A, FGF-2-GFP almost exclusively appeared in the soluble fraction (lane 1) of detergent-treated membranes. As control proteins, the Golgi-localized transmembrane protein p23 (Sohn et al., 1996) was used as a non-lipid raft marker (Gkantiragas et al., 2001) and the plasma-membrane-localized protein caveolin-1 was used as a classical lipid raft marker (Rothberg et al., 1992; Kurzchalia and Parton, 1999). Consistently, p23 could be detected only in the soluble fraction (lane 1), whereas caveolin was almost exclusively found in the insoluble fraction (lane 2). These results were further substantiated by the results from the flotation experiment performed with detergent-treated membranes. As shown in Fig. 9B, caveolin-1 could be detected in the light fractions [corresponding to about 15% (w/v) sucrose] of the flotation gradient. By contrast, both p23 and FGF-2-GFP were exclusively localized to the bottom fractions of the gradient demonstrating that the microdomains observed by confocal microscopy are not related to lipid rafts. A formal possibility would be that a potential association of FGF-2-GFP with lipid rafts cannot be detected because the interaction of FGF-2-GFP with cell surface HSPGs is detergent-sensitive which, in turn, would cause FGF-2-GFP to appear in the supernatant of detergent-treated membranes. However, as demonstrated in Fig. 2, FGF-2-GFP can be affinity-purified from cellular detergent extracts by using heparin-sepharose, a method that mimics the interaction of FGF-2 with heparan sulfate proteoglycans (Burgess and Maciag, 1989). Therefore, FGF-2-

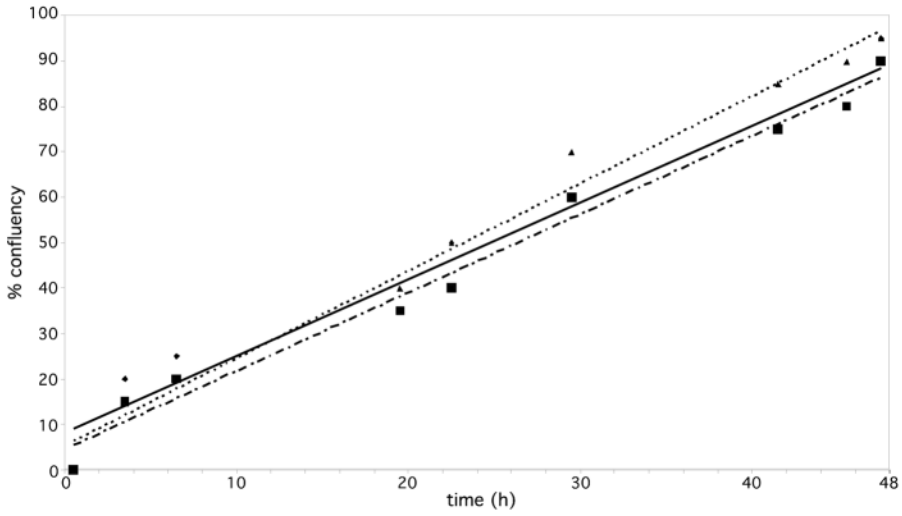

Fig. 10. Both endogenous and exogenously added FGF-2 do not stimulate proliferation activity of CHO cells. $CHO_{FGF\text{-}2\text{-}GFP}$ cells were spread on culture plates at a confluency of about 5-10%. Cell proliferation was monitored for 48 hours in the presence of recombinant His_6-FGF-2 (5 μg/ml; dotted line); in the presence of doxicyclin to induce FGF-2-GFP expression and externalization (solid line); or in the absence of doxicyclin as a control condition (dashed and dotted line).

GFP-positive microdomains found on the cell surface of $CHO_{FGF\text{-}2\text{-}GFP}$ cells are not related to lipid rafts.

Since FGF-2 signalling has been demonstrated to originate from caveolae-like lipid rafts (Davy et al., 2000), we analyzed whether exported FGF-2-GFP or exogenously added recombinant FGF-2 are able to stimulate cell proliferation. As shown in Fig. 10, neither secreted FGF-2-GFP nor recombinant FGF-2 added to the culture medium induce cell proliferation. These data are consistent with the fact that, despite expressing HSPGs and the FGF-2 co-receptor GM_1 (Rusnati et al., 2002), CHO wild-type cells do not express high-affinity FGF receptors (Rusnati et al., 2002). Therefore, FGF receptors appear to be required to target FGF-2 to caveolae-like lipid rafts involved in FGF signalling.

Intercellular spreading of exported biosynthetic FGF-2-GFP

To distinguish a translocation mechanism that involves a soluble intermediate between export and binding to proteoglycans from an integrated process where the export machinery directly delivers FGF-2 to the proteoglycan binding site, we conducted experiments where FGF-2-GFP-expressing cells were cultured together with cells lacking the FGF-2-GFP reporter construct ($CHO_{MCAT\text{-}TAM2}$ cells; see Materials and Methods). In this way we were able to analyze intercellular spreading of FGF-2 between different populations of CHO cells. As depicted in Fig. 11B,C, $CHO_{MCAT\text{-}TAM2}$ cells (labeled with an asterisk) not expressing the FGF-2-GFP fusion protein were found to be positive for cell-surface-localized FGF-2-GFP. To verify these observations using an independent approach we prepared a 100,000 $\boldsymbol{g}_{av}$ supernatant from doxicyclin-induced homogenized $CHO_{FGF\text{-}2\text{-}GFP}$ cells, which was added to $CHO_{MCAT\text{-}TAM2}$ cells, and analysed the cell surface staining using confocal microscopy. FGF-2-GFP appears in bright spots on the cell surface closely resembling the localization of secreted FGF-2-GFP (data not shown). These results demonstrate that, following secretion of FGF-2-

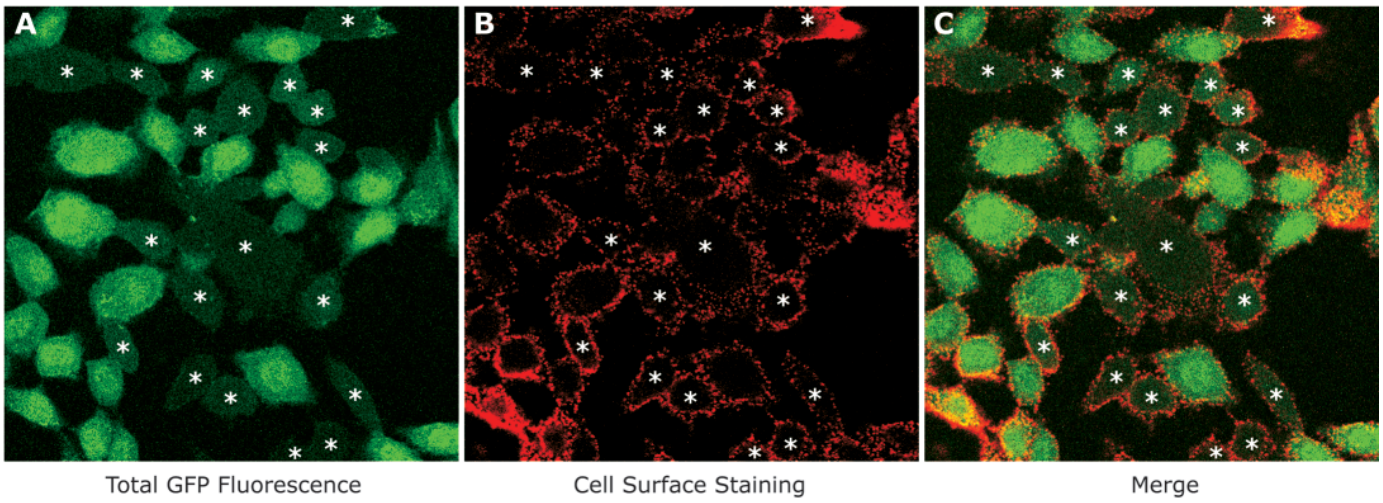

Fig. 11. Intercellular spreading of secreted FGF-2-GFP. $CHO_{FGF\text{-}2\text{-}GFP}$ and $CHO_{MCAT\text{-}TAM2}$ cells were cultured on glass coverslips in a 1:1 ratio. Following incubation for 24 hours at 37°C in the presence of doxicyclin, the cells were fixed with PFA and processed with affinity-purified anti-GFP antibodies. Primary antibodies were detected with anti-rabbit IgG antibodies coupled to Alexa546. The specimens were viewed using a Zeiss LSM 510 confocal microscope.

GFP, a soluble intermediate exists that subsequently accumulates in cell surface microdomains based on the interaction with HSPGs.

Discussion

The phenomenon of non-conventional protein secretion has been known for more than ten years (Cleves, 1997; Hughes, 1999); however, our knowledge about the molecular machinery mediating this process remains poor. It is even unclear as to whether the various proteins known to be secreted by non-conventional means make use of a common molecular mechanism (Hughes, 1999). In fact, evidence is accumulating that distinct machineries are in place; for example, the mechanism of IL1β secretion appears to involve intracellular vesicles (Andrei et al., 1999) whereas galectin secretion is likely to occur through plasma membrane blebbing (Mehul and Hughes, 1997). Another example are the distinct characteristics of FGF-1 versus FGF-2 secretion since FGF-1 export is sensitive to heat shock treatment (Jackson et al., 1992), whereas FGF-2 export is not (Mignatti et al., 1992). The relatively poor knowledge about the molecular components involved in theses processes compared to the advanced state of our knowledge about ER-Golgi-dependent protein secretion emphasizes the need for novel experimental strategies in order to reveal the molecular mechanism of non-conventional protein secretion.

In the first part of this study, we implemented a novel experimental system that will greatly facilitate studies on the molecular machinery of FGF-2 secretion. A key aspect was to reconstitute FGF-2 secretion in living cells based on a read-out method that provides a precise and quantitative analysis of this process. Moreover, by using FGF-receptor-deficient CHO cells, secondary effects based on FGF-2-induced signal transduction can be avoided. We have established genetically altered cell lines that stably express N- and C-terminally GFP-tagged FGF-2 in a doxicyclin-dependent manner. Moreover, these cells express the mouse orthologue of the cationic amino acid transporter [MCAT-1 (Albritton et al., 1989; Davey et al., 1997)], whose cell surface expression makes non-mouse cells permissive to ecotropic retroviruses (Albritton et al., 1989). This strategy allows the efficient retroviral transfer of cDNA libraries into MCAT-expressing mammalian cells of any origin. Following stable integration of the MCAT-1 cDNA using conventional methods, we took advantage of this approach by introducing both a doxicyclin-sensitive transactivator (Urlinger et al., 2000) and the various FGF-2-GFP cDNA constructs by using a retrovirus containing an ecotropic host-range envelope protein. In this way the cDNA constructs were stably integrated into the genomic DNA of the host cells without the need for cell selection based on antibiotics.

The resulting clonal cell lines can be transduced with retroviral particles containing specific cDNAs or cDNA libraries with high efficiency and express FGF-2-GFP fusion proteins in a strictly doxicyclin-dependent manner. These cells were functionally characterized with regard to non-conventional secretion of the FGF-2-GFP reporter molecules. Based on a robust and efficient FACS-based assay, it is possible to quantitatively assess the amount of FGF-2 released to the extracellular space in living cells. This is because, following its secretion, FGF-2-GFP binds to the extracellular surface of the plasma membrane, where it is associated with proteoglycans of the heparan sulfate type (Burgess and Maciag, 1989; Pellegrini et al., 2000; Trudel et al., 2000). This allows specific detection of secreted FGF-2-GFP with affinity purified anti-GFP- or anti-FGF-2 antibodies under native conditions based on flow cytometry. In this way, GFP-derived fluorescence is used to normalize the overall expression of the reporter molecule under various experimental conditions, whereas the secreted population can be exclusively detected on the cell surface with antibodies coupled to a second fluorophore such as phycoerythrin.

In the second part of this study, we made use of the experimental system described to study the fate of biosynthetic (i.e. endogenous) FGF-2-GFP following translocation to the extracellular compartment. FGF-2-GFP is shown to accumulate in large macromolecular clusters that appear as bright spots on the cell surface. FGF-2-GFP association with these structures is mediated by HSPGs, as heparin treatment causes a loss of FGF-2-GFP staining on the cell surface. To investigate whether FGF-2 export and deposition in HSPG-containing microdomains are tightly linked processes we analyzed the mode of delivery of FGF-2-GFP to HSPGs following its externalization. A soluble intermediate is demonstrated that allows FGF-2-GFP to spread between different populations of cultured cells. Moreover, FGF-2-GFP prepared as a cell-free supernatant from homogenized $CHO_{FGF\text{-}2\text{-}GFP}$ cells can associate with non-expressing cells thereby forming morphologically similar microdomains on their surfaces. In conclusion, FGF-2 externalization and deposition in cell surface microdomains do not occur through an integrated process that would restrict cell surface deposition to FGF-2-secreting cells. Rather, a soluble intermediate is released and eventually accumulates in HSPG-containing protein clusters. These data are consistent with our finding that the FGF-2 binding capacity mediated by HSPGs does not influence the balance of intracellular FGF-2 versus extracellular HSPG-bound FGF-2. Rather, the cell surface signal detected provides a precise measure of FGF-2 externalization that is not limited by the availability of HSPGs. Therefore, the FGF-2 export machinery is rate-limiting under the conditions applied.

The presence of FGF-2-GFP in discrete microdomains on the cell surface implied that these structures might represent protein complexes involved in FGF-2 signal transduction. In this context, Davy et al. reported that FGF-2 signalling originates from caveolae-like lipid rafts on the cell surface (Davy et al., 2000). A functional FGF-2 signal transduction complex consists of FGF-2, HSPGs, the co-receptor GM_1 and high-affinity FGF receptors (Rusnati et al., 2002). CHO wild-type cells do synthesize HSPGs and GM_1; however, they do not express high-affinity FGF receptors (Rusnati et al., 2002). Therefore, it was interesting to study the biophysical properties of the FGF-2-GFP-positive microdomains observed on the cell surface of CHO cells. Based on detergent solubility combined with flotation experiments in sucrose gradients, we can exclude that the FGF-2-positive clusters observed are related to lipid rafts. Therefore, initial binding of FGF-2 to HSPGs does not result in the correct targeting to caveolae-like lipid rafts, where FGF-2 signalling is initiated. Rather, FGF receptors are required to direct the core complex of FGF-2 signaling to lipid rafts. Accordingly, upon doxicyclin-induced FGF-2-GFP

expression and externalization, $CHO_{FGF\text{-}2\text{-}GFP}$ cells do not appear to be significantly stimulated with respect to cell proliferation. Therefore, the large FGF-2-GFP- and HSPG-containing cell surface clusters appear to represent signalling complex precursors that, in the presence of high affinity FGF receptors, are converted into functional signalling complexes. This transition appears to be accompanied by a targeting of the FGF-2 signalling complex to caveolae-like lipid rafts.

The FACS-based FGF-2 secretion assay described in this study is a powerful tool for the analysis of the molecular machinery mediating FGF-2 export. For example, the systematic testing of candidate proteins (e.g. identified by interaction studies or genetic screening in mammalian cells) can be carried out by transiently inhibiting their biosynthesis based on RNA interference (Elbashir et al., 2001). In this context, a considerable advantage of the FGF-2-GFP-based system is that total protein expression (GFP-derived fluorescence) and secreted FGF-2-GFP (PE-derived cell surface staining) can be measured independently. Therefore, a phenotype determined by PE-derived cell surface staining can be corrected by normalization based on the degree of total FGF-2-GFP expression. Due to the lack of FGF receptors in CHO cells, another unique feature of the experimental system described is the uncoupling of FGF-2 externalization from FGF-2 signalling. Therefore, FGF-2 export can be studied without the risk of secondary effects provoked by the action of the secreted product.

Another obvious application is a systematic high throughput screening for inhibitors (e.g. derived from natural compound libraries) of FGF-2 secretion and the subsequent functional identification of their cellular targets. Given the biological function of FGF-2 as a direct stimulator of tumor angiogenesis (Bikfalvi et al., 1997), inhibitors of FGF-2 secretion might have strong biomedical implications as potential lead compounds for the development of anti-angiogenic drugs.

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