Deletion mutant of FGFR4 induces onion-like membrane structures in the nucleus

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Summary

The expression of several deletion mutants of fibroblast growth factor receptor 4 (FGFR4) was studied in COS-1 cells. FGFR4-mutants lacking most of the extracellular region did not efficiently reach the plasma membrane but accumulated in the endoplasmic reticulum (ER) and Golgi body. A mutant FGFR4 lacking the kinase domain as well as most of the extracellular region (ΔExt/R4Tth) had a distinct intracellular distribution. It localized in part to the nucleus, where it exhibited a striking spotted pattern. Ultrastructural studies showed that the nuclear spots consisted of several layers of membrane that were folded into onion-like structures at the nucleoplasmic side of the nuclear envelope. These intranuclear structures did not contain nuclear pores but were positive for the ER proteins calreticulin and protein disulfide isomerase, in addition to abundant ΔExt/R4Tth. Formation of the intranuclear structures was sensitive to inhibition of protein kinase C. Live microscopy of a green-fluorescent-protein/ΔExt/R4Tth fusion protein showed that the intranuclear structures were stable and immobile, suggesting that they function as deposits of the overexpressed mutant and associated membrane. The ΔExt/R4Tth protein also induced formation of densely packed membrane stacks in the cytosol and we suggest a model were the intranuclear structures are formed by invagination of ER-derived membrane stacks into the nucleus.

Key words: ER, Golgi, Electron microscopy, Immunofluorescence confocal microscopy, Intracellular distribution, COS-1, Intranuclear

Introduction

Fibroblast growth factor receptors 1-4 (FGFR1-4) are high affinity receptors for the large family of fibroblast growth factors, which mediate a range of biological responses such as cell proliferation, differentiation and migration (Burgess and Maciag, 1989; Hughes, 1997; Ornitz and Itoh, 2001). The prototype FGFR is a single-pass type-I transmembrane protein with three extracellular immunoglobulin (Ig)-like domains involved in binding of ligand constituted by FGF-heparan complexes. The intracellular part of the receptor consists of a juxtamembrane region of roughly 75 amino acids, the split tyrosine kinase domain (approximately 275 amino acids) and a C-terminal tail of 55-65 amino acids. Several splice variants have been identified for FGFR1-4, including variants with distinct ligand specificities, soluble variants lacking the transmembrane region and isoforms with deletions in the intracellular region affecting kinase activity or the availability of docking sites for downstream effectors (Ueno et al., 1992; Yan et al., 1993; Wang and Thomas, 1994; Kishi et al., 1994; van Heumen et al., 1999; Powers et al., 2000; Ezzat et al., 2001; Terada et al., 2001; Burgar et al., 2002).

Binding of ligand induces dimerization of the receptors, followed by receptor autophosphorylation and the initiation of intracellular signaling cascades (reviewed in Klint and Claesson-Welsh, 1999). Examples of effectors that interact with FGFRs are FRS2, which binds to the juxtamembrane region of FGFR and recruits the Grb-Sos complex (Ong et al., 2000), and PLCγ, which binds in the C-terminal region of FGFR (Mohammadi et al., 1991).

In addition to signaling through the FGF receptors, it has been shown that FGF-1 and FGF-2 can traverse cellular membranes and enter the cytosol and nucleus. Nuclear localization is necessary for the full signaling and mitogenic potential of FGF (Imamura et al., 1990; Zhan et al., 1992; Zhan et al., 1993; Wiedlocha et al., 1994; Imamura et al., 1994; Wiedlocha et al., 1995; Wiedlocha et al., 1996). Recent studies suggest that translocation of FGF-1 occurs from an endosomal compartment possessing vacuolar proton pumps (Malecki et al., 2002). Binding to FGFR is a prerequisite for translocation of FGF, but the exact role of the receptor in the translocation process is not known.

To study the intracellular sorting of FGFR and FGF, we have earlier studied the uptake of FGF-1 in COS-1 cells transfected with FGFR4. We found that the complex of FGF-1 and FGFR4 was endocytosed and accumulated in a juxtanuclear region identified as the recycling endosomal compartment (Citores et al., 1999). Certain mutations of the receptor prevented it from reaching this compartment (Citores et al., 2001). A portion of the FGFR4 protein expressed in COS-1 cells was also found to be Triton X-100 insoluble and purified with the nuclei upon cell fractionation, presumably through linkage to cytoskeletal elements (Citores et al., 1999).

In this study, we have investigated deletion mutants of FGFR4 to study which parts of the receptor are required for its intracellular localization. A particular combination of deletions in the extracellular and intracellular regions resulted in a protein that localized to the ER and the inner nuclear membrane (INM), and also induced the formation of unusual

intranuclear membranous structures, which appears to function as deposits of the overexpressed mutant and associated membrane.

Materials and Methods

Materials

Rabbit anti-FGFR4 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-GM130 antibody and mouse anti-nucleoporin p62 antibody were from Transduction Laboratories (Lexington, KY). Mouse antibodies against protein-disulphide-isomerase (PDI) and calreticulin were from Stressgen Biotechnologies (Victoria, British Colombia, Canada). The secondary antibodies FITC-labeled anti-rabbit IgG and rhodamine-labeled anti-mouse IgG were from Jackson Immuno-Research Laboratories (West Grove, PA). Anti-Nopp140 antibody (Meier, 1996) was a gift of T. Meier (Albert Einstein College of Medicine of Yeshiva University, NY). 5-Bromo-2′-deoxyuridine (BrdU), anti-BrdU antibody and staurosporine were from Sigma (St Louis, MO). Brefeldin A (BFA) was from Epicenter Technologies (Madison, WI). Bisindolylmaleimide (BIM) and the transfection reagent Fugene-6 were from Boehringer Mannheim (Indianapolis, IN). H-89 was from BIOMOL Research Laboratories (Plymouth Meeting, PA). Complete EDTA-free Protease Inhibitor Cocktail Tablets were from Roche Applied Science (Penzberg, Germany). Protein-G/Sepharose and [^{33}P]-phosphate were from Amersham Pharmacia Biotech (Uppsala, Sweden). [^{35}S]-Methionine (EXPRE^{35}S^{35}S [^{35}S] Protein Labeling Mix) was from PerkinElmer Life Sciences (Boston, MA).

Plasmids

pcDNA3-R4, pcDNA3-R4ΔEag/*Bam*HI and pcDNA3-R4Tth have been described previously (Klingenberg et al., 2000). pcDNA3-ΔExt/R4 and pcDNA3-ΔExt/R4Tth were constructed by PCR amplification of a fragment of the pcDNA3-R4 and pcDNA3-R4Tth plasmids, respectively, using the forward primer 5′-TGTGGG-CGGGCTGAGCGAGCGCCCGAGGCCAGGTATACGGACATC-3′, which hybridizes to positions 1138-1165 in the *FGFR4* gene and has a 5′ tag with a restriction site for *Cel*II, and the reverse primer 5′-AATTTTATCCGAATGGAAATGAGCTG-3′, which hybridizes to the *FGFR4* gene downstream sequence. These PCR products were cut with *Cel*II and *Bam*HI (position 2423), and ligated into pcDNA3-R4 or pcDNA3-R4Tth cut with the same enzymes. The new extracellular sequence reads GRAER^{62}A^{362}PEAR. ΔExt/R4/ΔInt was constructed by PCR amplification of a fragment of pcDNA3-ΔExt/R4Tth using forward primer 5′-CTCTGAGGAAGTGGAGCT-3′ and reverse primer 5′-GGATCCTCGGCCGTGGAGCGCCT-3′. The reverse primer hybridizes to positions 1233-1248 and has a tag with a cut site for *Bam*HI. The PCR product was cut with *Blp*I (position 1154) and *Bam*HI, and ligated into pcDNA3-ΔExt/R4Tth cut with the same enzymes. The new intracellular sequence reads ALHGR^{398}G^{790}SSSF. The correct sequence of the plasmids was verified by sequencing. The plasmid pCDNA3-ΔExt-GFP/R4Tth encodes a protein in which green fluorescent protein (GFP) is inserted between amino acids 62 and 362 in ΔExt/R4Tth, and was constructed by a combination of PCR reactions. The upstream region of *FGFR4*, encompassing the coding region for amino acids 1-62, was amplified by the primers 5′-AC-CCACTGCTTACTGGCTT-3′ and 5′-TCGCTCAGCCCGCCCACA-3′. The coding region of GFP amino acids 1-239 was amplified from the pEGFP vector (Clontech Laboratories, CA, USA) by primers 5′-TGTGGGCGGGCTGAGCGAATGGTGAGCAAGGGCGAG-3′ and 5′-ATACCTGGCCTCGGGCGCCTTGTACAGCTCGTCCATG-3′, which contains tags complementary to ΔExt/R4Tth at the insertion site for GFP. The downstream region of ΔExt/R4Tth (from amino acid 362) was amplified by primers 5′-AGGCACAGTCGAGGCTGAT-3′ and 5′-GCGCCCGAGGCCAGGTAT-3′. These three PCR products were mixed, annealed and amplified in a fourth PCR reaction using primers 5′-ACCCACTGCTTACTGGCTT-3′ and 5′-AGGCACAG-TCGAGGCTGAT-3′. The final PCR product encoding ΔExt-GFP/R4Tth was cut with *Hin*dIII and *Eco*RI, and ligated into pCDNA3.

Cells and transfections

COS-1 cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin in a 5% CO_2 atmosphere at 37°C. Transient expression of the different receptors was induced by transfecting cells with pcDNA3 with appropriate insert using the Fugene-6 transfection reagent according to the procedure given by the company. The cells were analysed 24 hours after transfection.

Immunofluorescence microscopy

Transfected cells grown on coverslips were fixed with 3% paraformaldehyde in PBS for 15 minutes at room temperature. After washing, the remaining paraformaldehyde was quenched with 50 mM NH_4Cl in PBS and the fixed cells were permeabilized with 0.3% Triton X-100 in PBS for 4 minutes. The coverslips were then incubated in PBS containing 0.05% Saponin and the primary antibody at room temperature for 20 minutes, washed and then incubated with secondary antibody. In some experiments, the cells were incubated with BFA (2 μg ml^{-1} for 3 hours), cycloheximide (10 μg ml^{-1} for 2 hours), BrdU (100 μM for 18 hours) or the Ser/Thr kinase inhibitors BIM (10-20 μM) or H-89 (20-40 μM) for 18 hours, before fixation as above. After staining, the coverslips were mounted in Mowiol and examined with a Leica (Wetzlar, Germany) confocal microscope.

Metabolic labeling of receptors

Cells transfected with receptors were starved in methionine free medium for 1 hour, labeled with [^{35}S]-methionine for 1 hour in the presence of 2 μg ml^{-1} BFA, washed and incubated further in normal medium with 2 μg ml^{-1} BFA. Cells were lysed at 0 hours, 2 hours or 4 hours after labeling. Receptors were immunoprecipitated from the lysate using rabbit anti-FGFR4 antibodies and Protein-A/Sepharose, analysed by sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and phosphor-imager scanning, and quantified using ImageQuant 5.0 software.

Electron microscopy

Transfected COS-1 cells were fixed in a mixture of 4% formaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (Na_2HPO_4, NaH_2PO_4, pH 7.4), gently scraped off the culture dish and pelleted by centrifugation for 30 seconds at 13,000 rpm in a bench top centrifuge. The resulting pellet was sliced into small blocks and infused with 10% polyvinylpyrrolidone/2.3 M sucrose overnight at 4°C before mounting and freezing in liquid nitrogen. Ultrathin cryosections were cut at –110°C on a Leica Ultracut and collected with a 1:1 mixture of 2% methylcellulose and 2.3 M sucrose. Sections were transferred to formvar/carbon-coated grids and labeled with primary antibodies followed by Protein-A/gold conjugates essentially as described by Slot et al. (Slot et al., 1991). Sections were observed in a Phillips CM100 electron microscope. For plastic embedding, cells were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer. Cells were scraped off the culture dish, pelleted in a microfuge at 13,000 rpm for 5 minutes and post-fixed with 2% OsO_4 and 1.5% KFeCN in the same buffer. Samples were then stained en bloc with uranyl acetate, dehydrated in graded ethanol series and embedded in Epon. Ultrathin sections were post-stained with lead citrate and observed in a Philips CM 10 microscope.

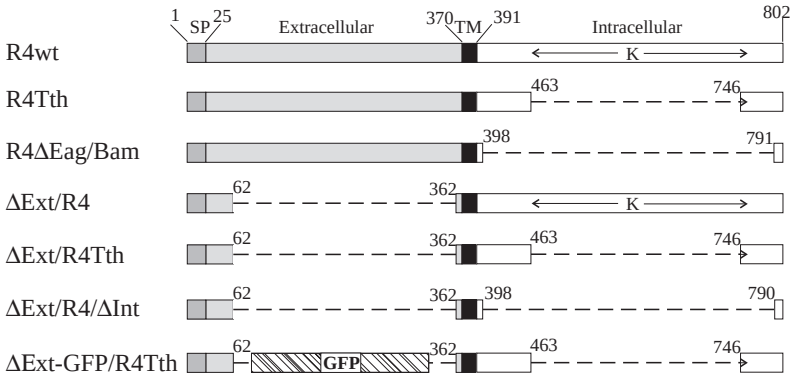

Fig. 1. FGFR4 and deletion mutants. The extracellular part of FGFR4 is indicated in gray and the intracellular part in white. Numbers refer to amino acid positions and stippled lines indicate the part of the receptor that has been deleted. GFP, green fluorescent protein; SP, signal peptide; TM, transmembrane region. Arrows and 'K' indicate the position of the tyrosine kinase domain.

FRAP experiments

COS-1 cells were seeded onto LabTek II tissue culture plates and transfected with pCDNA-ΔExt-GFP/R4Tth. The cells were studied after approximately 18 hours using a Zeiss LSM510 META confocal microscope equipped with an argon laser. During microscopy, the cells were kept in an integral incubator equipped with temperature and CO_2 control (at 37°C and 5% CO_2). Images were taken using a 63× Plan-Apochromat objective equipped with objective heater. A region of the nucleus was selectively photobleached by scanning for 60 cycles at maximum power. After the photobleaching pulse, the recovery of fluorescence in the cells was recorded by time-lapse confocal microscopy.

[^{33}P]-Labeling of receptors

Transfected COS-1 cells were incubated with [^{33}P]-phosphate in phosphate-free DMEM for 60 minutes in the absence or presence of the kinase inhibitors, BIM (5-20 μM), H-89 (10-40 μM) or staurosporine (1 μM). Then the cells were washed in PBS and dissolved by lysis in lysis buffer (0.1 M NaCl, 10 mM Na_2HPO_4, 1% Triton X-100, 1 mM EDTA, pH 7.4, supplemented with complete protease inhibitors) followed by dilution in 0.5 M NaCl and sonication. The receptors were immunoprecipitated from the solution by anti-FGFR4 antibodies and Protein-G/Sepharose. After washing of the Sepharose beads with PBS containing 0.1% Tween 20, bound protein was eluted by heating in SDS sample buffer and then analysed by SDS-PAGE and autoradiography.

Results

Intracellular distribution of wild-type and deleted forms of FGFR4

To study the role of different parts of the FGFR4 molecule in regulating its intracellular distribution we made several deletion mutants of the receptor (Fig. 1). In all cases, the mutant receptors retained the signal sequence and the transmembrane part to ensure membrane insertion, and the C-terminal 12 amino acids that constitute an epitope recognized by the anti-FGFR4 antibody used in these studies.

FGFR4 wild-type receptor (R4wt) and the mutants were transiently transfected into COS-1 cells and the cells were fixed, stained with anti-FGFR4 and FITC-labeled secondary antibodies, and examined by confocal fluorescence microscopy. Fig. 2 demonstrates that full-length R4wt and mutants with deletions in the intracellular (cytosolic) region (R4Tth and R4ΔEag/*Bam*HI) were expressed on the plasma membrane as well as in intracellular compartments

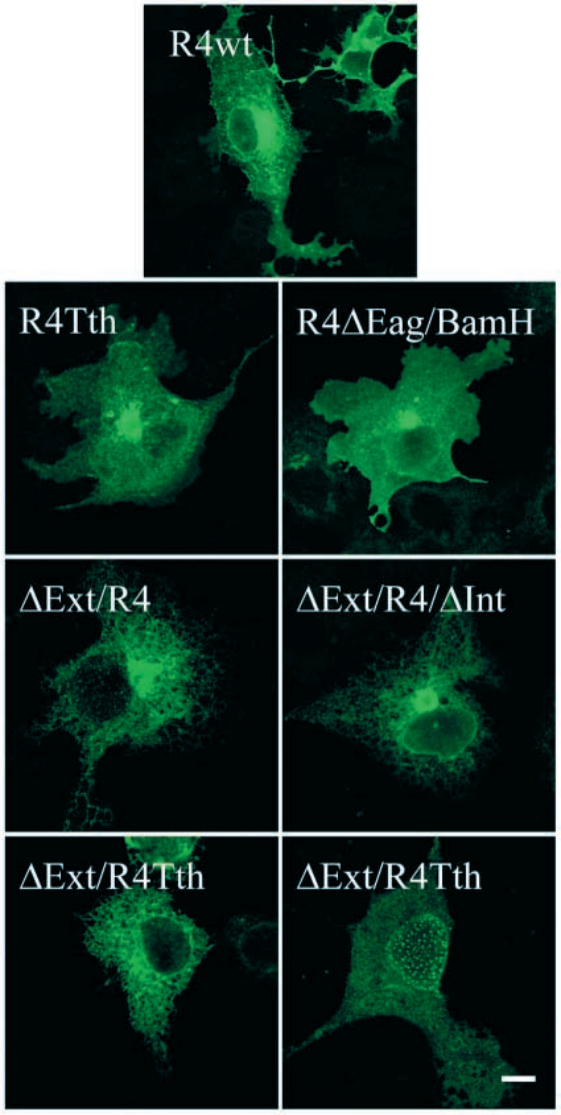

Fig. 2. Intracellular localization of full-length and deletion mutants of FGFR4 in transfected COS-1 cells. Cells transfected with R4wt, R4Tth, R4ΔEag/*Bam*HI, ΔExt/R4, ΔExt/R4/ΔInt and ΔExt/R4Tth, as indicated, were fixed, labeled with anti-FGFR4 antibodies and FITC-conjugated secondary antibodies, and analysed by confocal microscopy. Two examples of cells transfected with ΔExt/R4Tth are shown. Scale bar, 10 μm.

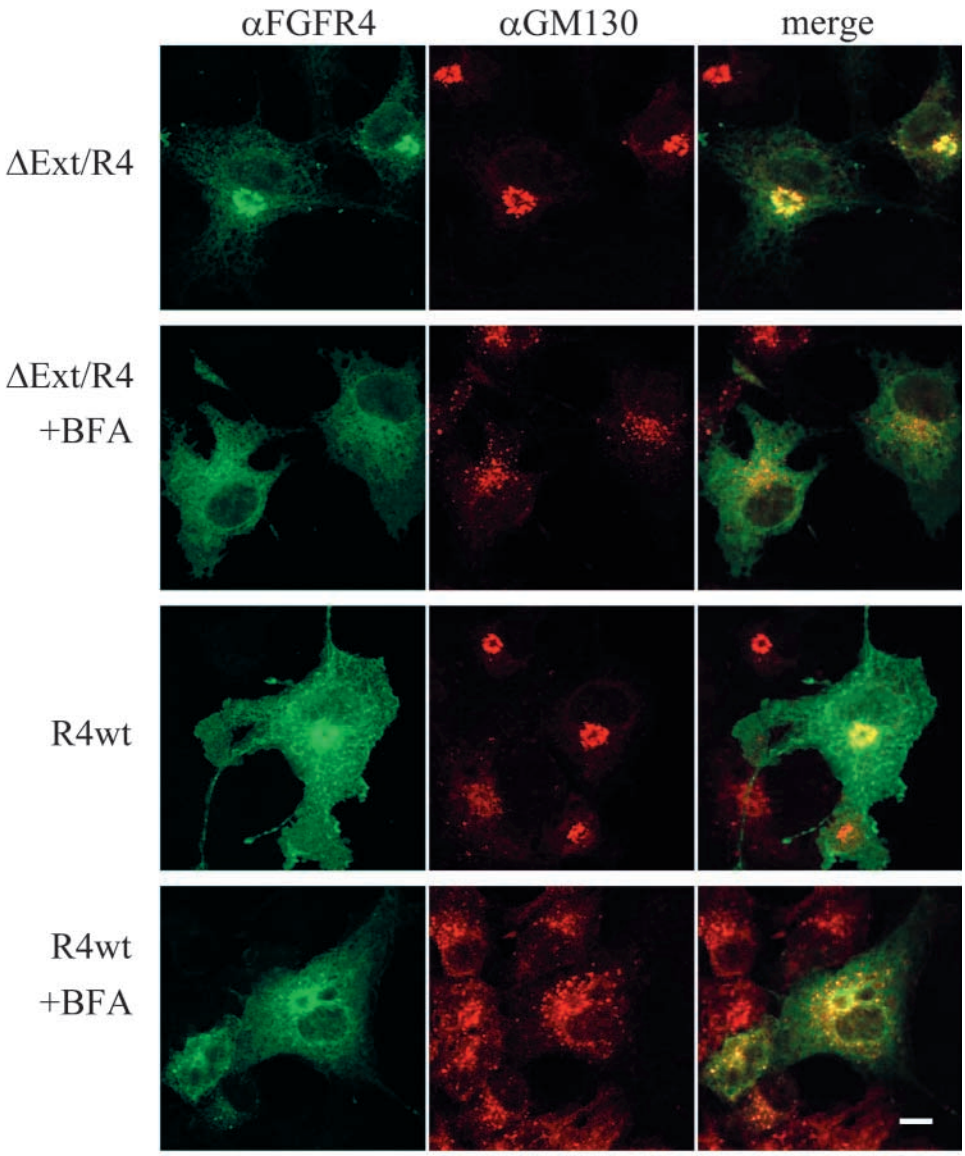

Fig. 3. ΔExt/R4 localizes with the Golgi marker GM130 in a BFA-sensitive juxtanuclear region, whereas R4wt localizes to a BFA-resistant juxtanuclear compartment. COS-1 cells transfected with ΔExt/R4 (first and second rows) or R4wt (third and fourth rows) were fixed after pretreatment with 2 μg ml^{-1} BFA for 3 hours (second and fourth rows) or without BFA (first and third rows). The cells were double labeled with anti-FGFR4 antibodies and secondary FITC-conjugated antibodies (first column) and with anti-GM130 antibodies and rhodamine-conjugated secondary antibodies (second column), and analysed by confocal microscopy. Yellow color in the merged images (third column) indicates co-localization. Scale bar, 10 μm.

corresponding to ER, Golgi and vesicular membranes. Usually, very strong labeling of a juxtanuclear region was observed. We have previously demonstrated that, after binding of FGF-1, the receptor and ligand are transported to this region, which localizes with endocytosed transferrin and is therefore interpreted as the recycling endosomal compartment (Citores et al., 1999). R4ΔEag/*Bam*HI had somewhat more pronounced plasma membrane localization than R4wt. This is probably related to its inefficient endocytosis (Citores et al., 2001), leading to accumulation in the plasma membrane.

Receptor mutants in which most of the extracellular region had been deleted (ΔExt/R4, ΔExt/R4Tth and ΔExt/R4/ΔInt) were not found in significant amounts on the plasma membrane but localized mainly to a juxtanuclear region and to ER-like structures (Fig. 2) that co-stained for the ER marker PDI (data not shown).

When cells expressing receptors were treated with BFA, which disrupts the Golgi network, and co-stained for the Golgi protein GM130, we found that the juxtanuclear staining with R4wt was only partially dissolved, whereas the juxtanuclear staining with mutants deleted in the extracellular region was completely dissolved by the drug (shown for R4wt and ΔExt/R4 in Fig. 3). This indicates that wild-type receptor was transported to both the plasma membrane and the juxtanuclear, endosomal recycling compartment, which is BFA resistant and partially overlaps with the Golgi apparatus in the juxtanuclear region. Receptors lacking the extracellular region were inefficiently transported through the secretory pathway and therefore accumulated in the ER and Golgi apparatus.

Nuclear localization of ΔExt/R4Tth

The intracellular distribution of the ΔExt/R4Tth mutant was distinct from that of ΔExt/R4/ΔInt and ΔExt/R4. In many cells, this receptor mutant was localized mainly to the ER (reticulate structures, not the dense juxtanuclear GM130-positive compartment) but, in some cells, the protein was associated with the nucleus, where it exhibited a striking spotted pattern (Fig. 2, bottom). The number of cells that showed this pattern varied from experiment to experiment but, typically, 10-20%

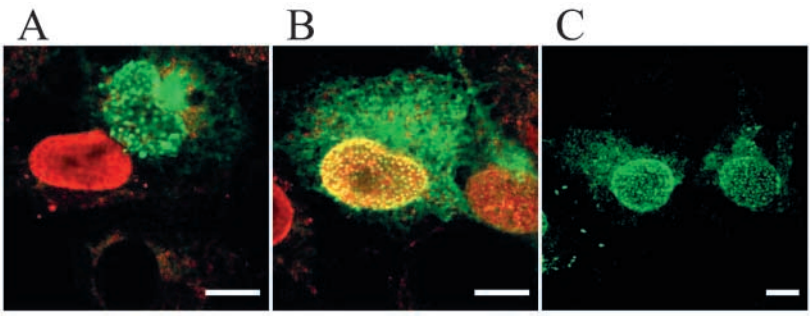

Fig. 4. Nuclear structures induced by ΔExt/R4Tth are found in cells at various cell cycle stages. (A,B) COS-1 cells transfected with ΔExt/R4Tth were incubated with BrdU for 18 hours, then fixed and labeled with anti-BrdU (red) and anti-FGFR4 (green) antibodies. Yellow color indicates overlap between red and green signal. The ΔExt/R4Tth-transfected cell has not incorporated BrdU in A, whereas the transfected cell in B has. (C) Nuclear spots could be observed in pair of cells, indicating that they arose by division of a single transfected cell. Scale bar, 10 μm.

of the transfected cells exhibited nuclear spots, and these structures occurred in cells with both relatively low and high expression levels of ΔExt/R4Tth. A similar pattern for ΔExt/R4Tth expression could be observed also by transient transfection of other cell types such as U2OS and HeLa (not shown) and is therefore not limited to COS-1 cells.

To investigate whether formation of these structures was dependent on a particular cell cycle stage, we incubated the transfected cells with BrdU for 18 hours before staining with anti-BrdU and anti-FGFR4 antibodies. This showed that cells with nuclear spots had sometimes gone through S-phase and sometimes not (Fig. 4A,B), in a ratio similar to that of untransfected cells. Transfected cells possessing nuclear spots could also be observed in pairs, indicating that they arose by the division of a single transfected cell (Fig. 4C). Optical sections by confocal microscopy of the spotted nuclei showed that the spots were usually close to the nuclear envelope (NE) and no spots appeared to be in the center of the nucleus (Fig. 5A). Occasionally, spots were also seen in the cytoplasm (Figs 4, 5). Although the spots were found close to the NE, they did not seem to consist of conventional NE, because double labeling showed no strong co-localization with nucleoporin p62, a component of nuclear pores (Fig. 5B). Furthermore, the spots did not co-localize with SUMO-1 or PML, which can be found in subnuclear structures, or with Nopp140, a nuclear protein that has been shown to form similar nuclear spots (data not shown). However, PDI (a lumenal ER protein) localized with the receptor in the nuclear spots and in the ER (Fig. 5C).

To test whether the nuclear spots were a general response to accumulation of abnormal proteins that the cells were unable to secrete or degrade, we treated cells expressing R4wt, R4Tth or ΔExt/R4Tth with monensin or chloroquine (200 μM), which inhibits lysosomal protein degradation, or with lactacystin (10-25 μM), which inhibits proteasomal protein degradation. Although extended treatment (up to 15 hours) with these inhibitors eventually led to the accumulation of irregular structures labeled with anti-FGFR4 (possibly aggresomes) in the cells, we did not observe formation of nuclear spots by R4wt or R4Tth, nor did the treatment alter the general appearance or the fraction of ΔExt/R4Tth-expressing cells that possessed nuclear spots (data not shown). We also compared the stability of R4wt, R4Tth and ΔExt/R4Tth in the ER in a metabolic-labeling/pulse-chase experiment (Fig. 6). Because these proteins might have different transport rates out of the ER, the cells were treated with BFA to block transport out of the ER in this experiment for better comparison. By quantifying the

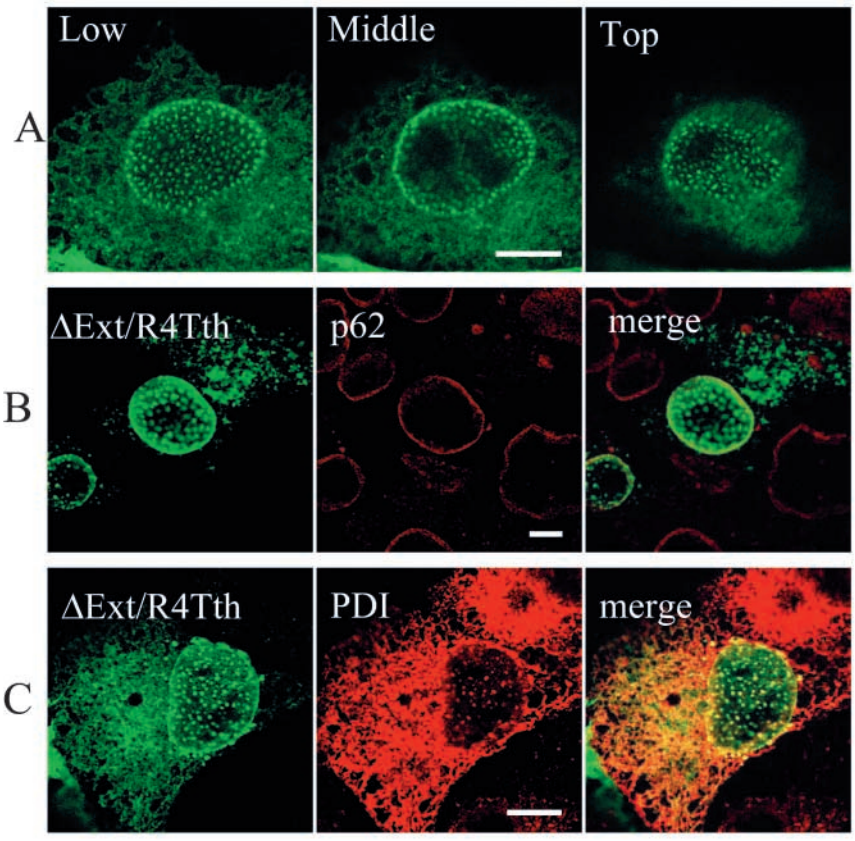

Fig. 5. ΔExt/R4Tth forms spots along the NE that stain for protein disulfide isomerase. COS-1 cells transfected with ΔExt/R4Tth were fixed, labeled with antibodies and examined by confocal microscopy. (A) Three sections of a cell labeled with anti-FGFR4 antibodies and FITC-conjugated secondary antibodies. (Low) Image taken close to the coverslip. (Middle) Image taken through the middle of the nucleus. (Top) Image taken at the top of the cell/nucleus. (B) Double labeling with anti-FGFR4 (green) and anti-nucleoporin p62 (red) antibodies. (C) Double labeling with anti-FGFR4 (green) and anti-PDI (red) antibodies. Yellow color indicates the overlap of red and green signal. Scale bar, 10 μm.

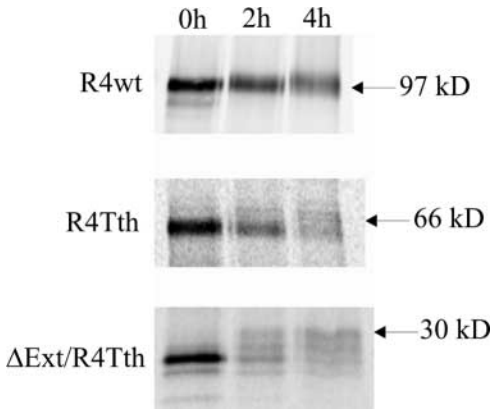

Fig. 6. ΔExt/R4Tth, R4Tth and R4wt have similar stability in the ER. COS cells transfected with R4wt, R4Tth or ΔExt/R4Tth were treated with BFA, metabolically labeled with [^{35}S]-methionine for 1 hour and then lysed after 0 hours, 2 hours or 4 hours, as indicated. Receptor was immunoprecipitated with anti-FGFR4 antibody, separated by SDS-PAGE and scanned using a phosphorimager.

radioactive signal in the gel, we found no large differences between the degradation rates of these proteins. In several similar experiments, 20-40% of R4wt, R4Tth or ΔExt/R4Tth remained in the cell after 4 hours. Thus, overexpression and intracellular accumulation alone cannot explain the formation of nuclear spots by ΔExt/R4Tth, but this must be attributed to other features of the protein. From the experiment shown in Fig. 6, it appeared that the ΔExt/R4Tth protein underwent post-translational modification that slowed down its migration in the gel. This modification did not consist of ubiquitylation or SUMOylation, because the shift in the migration was too low and the bands were not recognized by antibodies against ubiquitin or SUMO in a western blot (data not shown), but the modification could be due to phosphorylation (see Fig. 10).

EM studies of cells expressing wild-type and deleted forms of FGFR4

To study in more detail the localization of the receptors expressed in COS-1 cells, immunoelectron microscopy was carried out. We found that R4wt localized to membranes corresponding to ER, Golgi, vesicles, the plasma membrane and the outer nuclear membrane (ONM) (data not shown). To some extent, we also observed R4wt in the INM (Fig. 7A). We were, however, not able to identify a targeting motif in FGFR4 for INM localization, because this could be observed for all deletion mutants of FGFR4 and also for FGFR4 that had its transmembrane domain exchanged with the transmembrane domain from EGFR (not shown). Also, by overexpressing transferrin receptor or epidermal growth factor receptor (EGFR) in COS-1 could we occasionally observe these receptors in the INM by immunogold staining. Therefore, the INM localization could be a result of overexpression, presumably owing to free diffusion of proteins from the ER/ONM over the nuclear pores, consistent with existing models for transport of transmembrane proteins into the INM.

For the ΔExt/R4Tth mutant, by contrast, localization to the INM was observed frequently (Fig. 7B,C,E). EM studies also revealed that the spotted nuclear pattern of ΔExt/R4Tth observed by confocal microscopy in fact represented intranuclear onion-like structures containing several layers of membranes folded over each other in rather dense structures (Fig. 7C-F). The intranuclear membrane sometimes appeared to be ordered into concentric or spiral-like structures (Fig. 7C,D) and sometimes appeared to contain several internal membrane cisternae (Fig. 7E,F). The intranuclear structures were devoid of nuclear pores but contained, in addition to abundant ΔExt/R4Tth protein, the ER proteins calreticulin (Fig. 8A) and PDI (Fig. 8B). These structures therefore resemble the intranuclear structures previously described for Nopp140 (Isaac et al., 2001). In some cases, similar membrane stacks positive for PDI were found in the cytoplasm (Fig. 9A) but, notably, the cytoplasmic structures consisted of more elongated membrane stacks (Fig. 9B). Furthermore, membrane irregularities in the NE region could sometimes be observed as tubular structures consisting of several layers of membrane that were invaginated into the nucleus (Fig. 9C).

EM studies also revealed that some of the ΔExt/R4, ΔExt/R4/ΔInt and ΔExt/R4Tth proteins were localized to the plasma membrane (data not shown), indicating that transport through the secretory pathway was not completely blocked for these mutants. We did not observe cytosolic aggresomes or aggregates of the expressed protein for any of the wild-type or deleted FGFR4 mutants. All the receptor constructs were consistently associated with membranes, as expected for transmembrane proteins.

Formation of nuclear spots by ΔExt/R4Tth depends on protein kinase C

Nopp140 was reported to induce intranuclear folds due to a sequence rich in positively charged amino acids as well as phosphorylated serines (Isaac et al., 2001). The intracellular part of FGFR4 contains two serine-rich regions, which were brought into close proximity by deletion of the kinase domain in ΔExt/R4Tth. ΔExt/R4Tth has a total of 27 serines, several of which are potential protein kinase C (PKC) or protein kinase A (PKA) substrates.

We investigated whether the ΔExt/R4Tth receptor mutant was phosphorylated by labeling of transfected cells with [^{33}P]-phosphate followed by immunoprecipitation of the receptor and SDS-PAGE. This showed that ΔExt/R4Tth was phosphorylated (Fig. 10, first lane), as was R4wt and R4Tth (data not shown). Because all but one cytoplasmic tyrosine residue are deleted in ΔExt/R4Tth and the mutant has no kinase activity, the phosphorylation is likely to be mainly on serine residues.

To test whether phosphoserine is required to form the nuclear spots, we incubated ΔExt/R4Tth-transfected cells in the presence of H-89, an inhibitor of PKA, or with BIM, an inhibitor of PKC. We found that, by incubating with 10 μM BIM for 18 hours, the frequency of cells with nuclear spots was reduced from 15% (control cells) to less than 1% without significant reduction in cell viability or the expression level of the protein.

Similar incubation of the cells with up to 20 μM H-89 had no effect, whereas higher concentrations were toxic to the cells. When the phosphorylation status of the ΔExt/R4Tth protein was tested, we found, however, that neither BIM nor H-89 alone significantly reduced the phosphorylation of ΔExt/

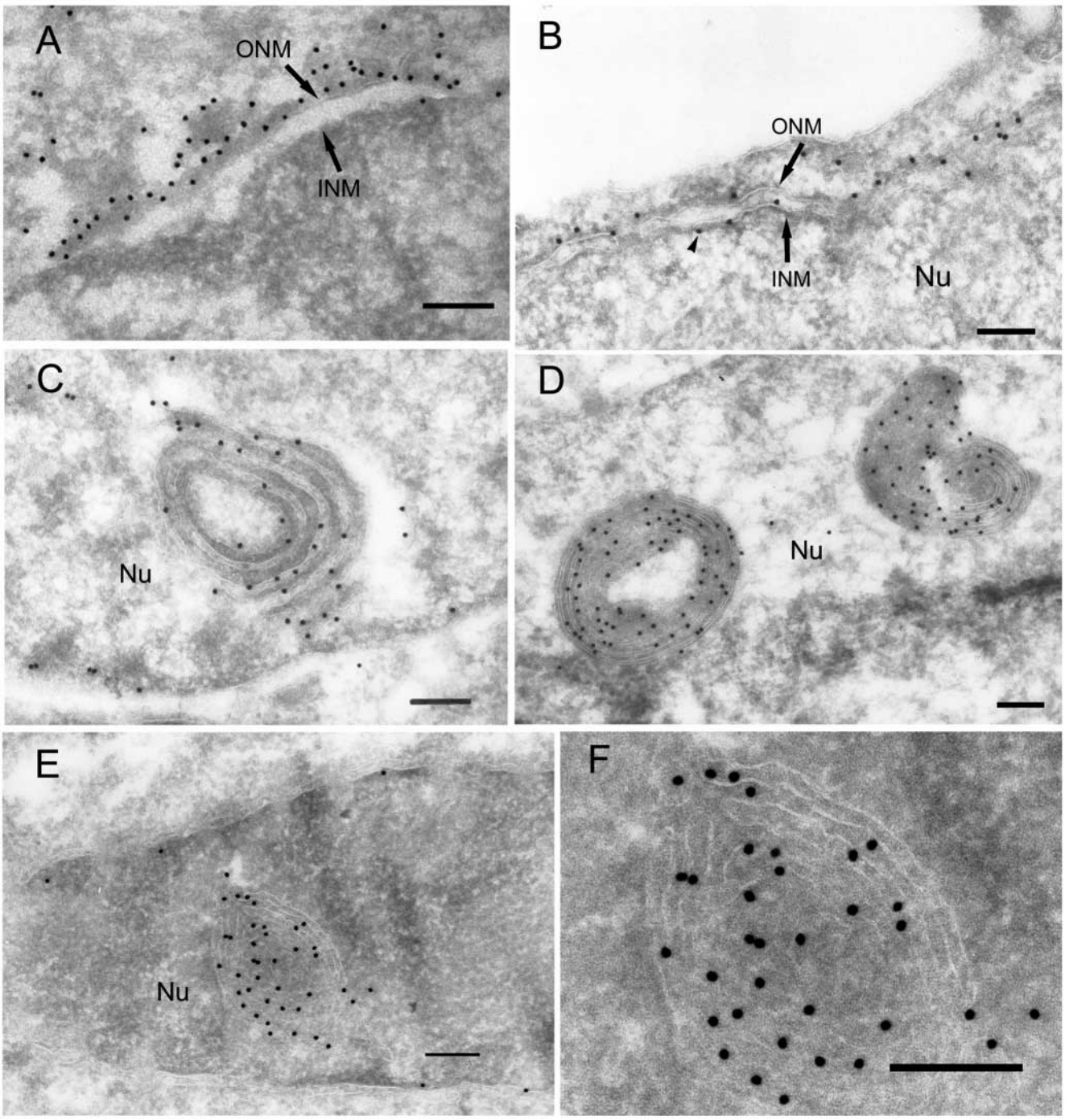

Fig. 7. ΔExt/R4Tth-induced intranuclear membranous structures. COS cells expressing R4wt (A) or ΔExt/R4Tth (B-F) were labeled with antibody against FGFR4 followed by protein-A/gold. (A) R4wt localized mainly to the outer membrane of the NE. (B) ΔExt/R4Tth localized to the NE. Labeling of both the INM and ONM can be seen. (C-F) ΔExt/R4Tth was found in the nucleus in onion-like membrane structures. (F) is a magnification of the central region of the image in E). Nu, nucleus. Scale bars, 200 nm.

R4Tth. By contrast, the combined action of BIM and H-89, or staurosporine, a protein kinase inhibitor with broad specificity, reduced its phosphorylation (Fig. 10). Thus, ΔExt/R4Tth can apparently be phosphorylated by several different Ser/Thr kinases and the inhibitory effect of BIM on the formation of intranuclear structures could be due to inhibition of phosphorylation of accessory proteins.

Because phosphorylation was necessary for the formation of nuclear spots, it was possible that inhibition of phosphatases would enhance the formation of nuclear spots. We therefore incubated cells expressing ΔExt/R4Tth with an inhibitor of Ser/Thr phosphatases (okadaic acid) for up to 18 hours, but no increase in the occurrence of nuclear spots could be observed.

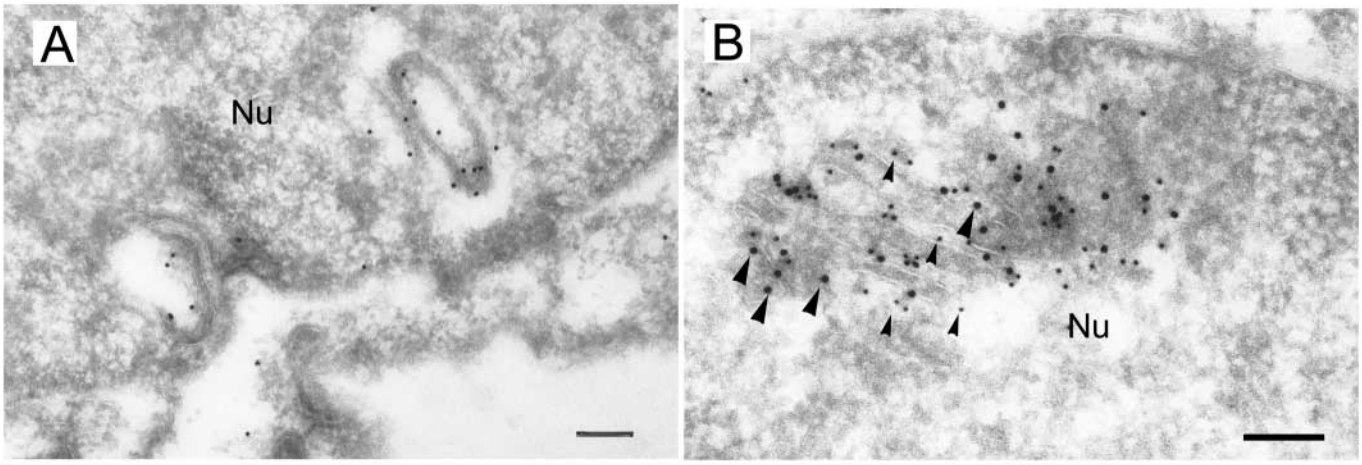

Fig. 8. The intranuclear membrane structures contain ER markers. COS cells expressing ΔExt/R4Tth were labeled with antibody against calreticulin and protein-A/gold (A), or double labeled for PDI (small arrowheads) and ΔExt/R4Tth (large arrowheads) (B). Nu, nucleus. Scale bars, 200 nm.

Live microscopy of a GFP-ΔExt/R4Tth fusion protein

To study the dynamics of the intranuclear structures induced by ΔExt/R4Tth, we constructed a GFP fusion protein. Our first attempt of adding the GFP protein to the C-terminus of ΔExt/R4Tth was unsuccessful, because this protein did not form any intranuclear structures. However, when GFP was inserted into the short 'extracellular' part of ΔExt/R4Tth (between amino acids 62 and 362) to yield the construct ΔExt-GFP/R4Tth, protein that showed an intracellular distribution similar to that of ΔExt/R4Tth was obtained, although the proportion of transfected cells that had intranuclear spots was reduced to about 1%.

Live microscopy of cells expressing ΔExt-GFP/R4Tth showed that the intranuclear structures (spots) were highly immobile structures, as opposed to cytoplasmic, vesicle-like structures containing ΔExt-GFP/R4Tth that were highly mobile. When the fluorescent signal in a part of the cell was bleached, recovery of the signal in the cytoplasm could usually be detected within a few minutes. In the nucleus, by contrast, no recovery of signal from spots could usually be seen even after 2-3 hours, although there were exceptions to this and, in some cases, partial recovery could be seen within 1 hour (Fig. 11). For cells that showed recovery of nuclear spots, this could be observed either as slowly emerging signal from immobile spots resembling spots that existed before bleaching (Fig. 11A) or as movement of strongly fluorescent spots from the juxtanuclear region and into the nuclear region (Fig. 11B). Spots that moved in from the juxtanuclear region, however, remained mobile and could represent a different type of structure than the immobilized nuclear spots.

We were not able to detect a reduction in the recovery of nuclear spots by acute treatment with the protein kinase inhibitor staurosporin, suggesting that ongoing phosphorylation is not required for the formation of intranuclear onion membrane. On the other hand, no recovery of nuclear spots could be observed when the cells had been pretreated with staurosporin for 4 hours.

Discussion

In this study, we describe several deletion mutants of FGFR4 that were constructed to investigate which part of the FGFR4 molecule is required for localization to various subcellular locations. These mutants were transiently expressed in COS-1 cells. The general observations were that mutants with large deletions in the intracellular region were expressed on the plasma membrane and also internalized to the recycling endosomal compartment, whereas mutants with a large deletion in the extracellular/ER-lumenal region did not efficiently reach the plasma membrane but accumulated in the ER and Golgi.

One of the mutants, ΔExt/R4Tth, which lacks most of the extracellular region as well as the intracellular kinase domain, was found to exhibit a very unusual pattern and was therefore studied in more detail. ΔExt/R4Tth accumulated in the ER and, by EM, we found that it also localized to the INM. Most strikingly, this mutant also induced the formation of intranuclear structures, which were observed as nuclear spots by confocal microscopy and were revealed by EM to consist of onion-like membranous structures on the nucleoplasmic side of the NE.

Localization of receptor in the INM

The INM is a specialized membrane domain with a unique protein composition (Georgatos, 2001; Holaska et al., 2002). According to the diffusion-retention model, there is free diffusion of transmembrane proteins from the ONM, which is continuous with ER, over the nuclear pore membrane into the INM, whereas only proteins that are specifically retained by interaction with nuclear components will actually accumulate in the INM. A targeting signal for INM localization has been shown to reside in the nucleoplasmic part of several proteins, such as the lamin B receptor (Soullam and Worman, 1993; Soullam and Worman, 1995), LAP2 (Furukawa et al., 1995; Furukawa et al., 1998), emerin (Ostlund et al., 1999) and MAN1 (Wu et al., 2002). For some proteins, the transmembrane region has been found to mediate INM localization; these include the lamin B receptor (Smith and Blobel, 1993; Soullam and Worman, 1995) and nurim (Rolls et al., 1999). Often, the targeting region overlaps with binding sites for nuclear lamina or chromatin. The diffusion over the

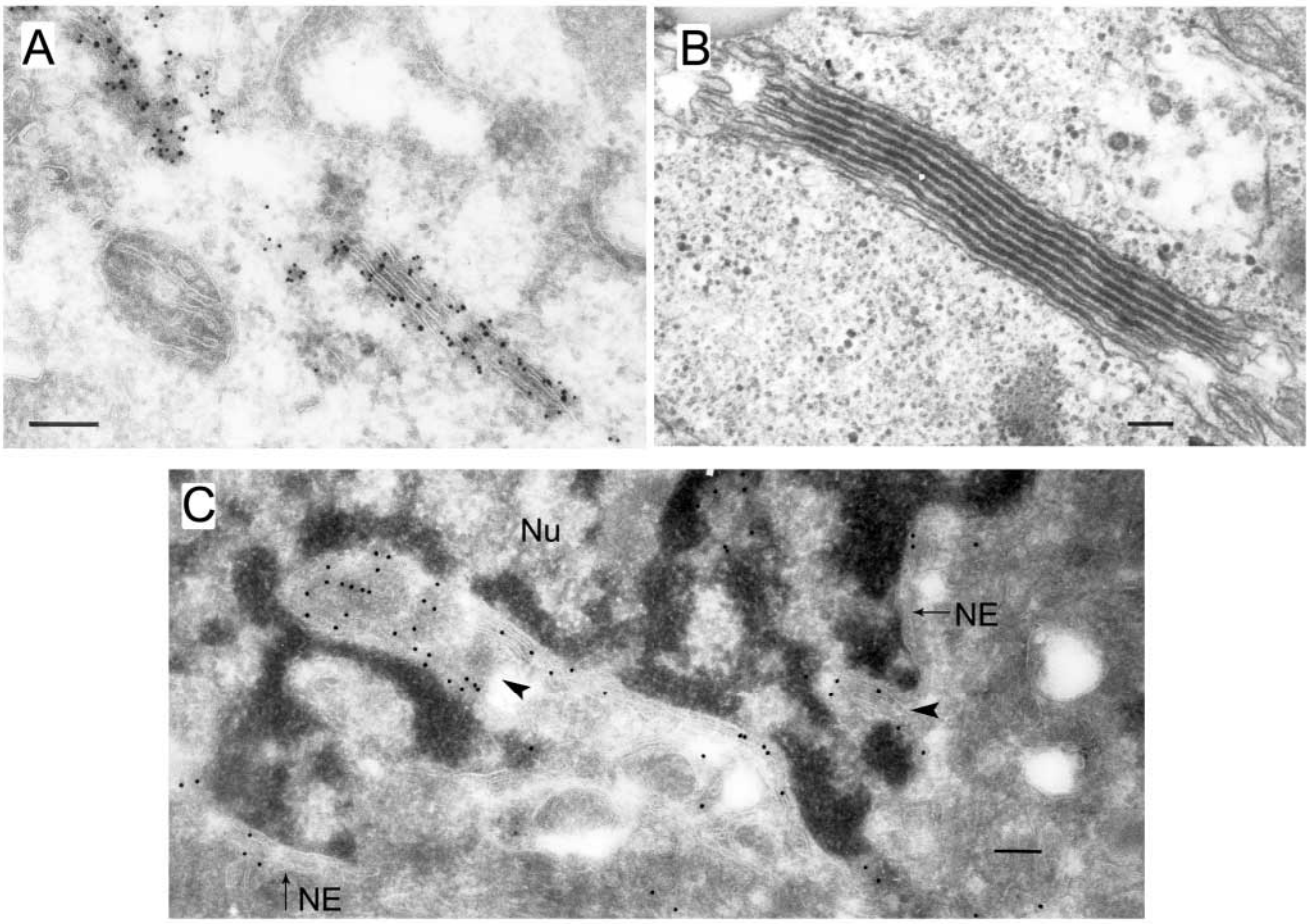

Fig. 9. Cytosolic membrane stacks in COS-1 cells transfected with ΔExt/R4Tth. (A) The cytosolic membrane stacks were positive for PDI (small gold particles) and ΔExt/R4Tth (large gold particles). (B) Cytosolic membrane stack showing its flattened dense structure (plastic-embedded material). (C) Invaginations into the nucleus (arrowheads) were positive for ΔExt/R4Tth (immunogold labeling) and contained several layers of membrane. Nu, nucleus. Scale bars, 200 nm.

nuclear pore membrane seems, however, to be restricted to proteins possessing nucleoplasmic domains of less than approximately 60 kDa, although it is possible that other features of the protein can also play a role (Smith and Blobel, 1993; Soullam and Worman, 1995; Ellenberg et al., 1997; Wu et al., 2002).

When FGFR4 and deletion mutants of this receptor were expressed in COS-1 cells, we could, to some extent, see by EM the protein in the INM. The molecular weight of full-length FGFR4 is approximately 110 kDa, of which the intracellular region constitutes approximately 57 kDa. This protein might therefore be sufficiently small to diffuse freely into the INM. We were unable to define an INM targeting motif in the FGFR4 receptor; mutants deleted in both the intracellular and the extracellular regions, and a mutant that had its transmembrane domain exchanged for that of EGFR all partly localized to the INM. Thus, it is possible that these proteins enter the INM by passive diffusion from the ER and are not efficiently cleared from this location owing to their high expression level, which gives a high protein concentration in the ER.

The mutant ΔExt/R4Tth differed from wild-type FGFR4 and the other mutants described here by being more frequently localized to the INM. This mutant was inefficiently secreted to the plasma membrane and accumulated mainly in the ER, possibly explaining why it was more abundant also in the INM.

Formation of onion-like structures in the nucleus

In a proportion of the cells expressing ΔExt/R4Tth (10-20%, apparently irrespective of the expression level or cell cycle

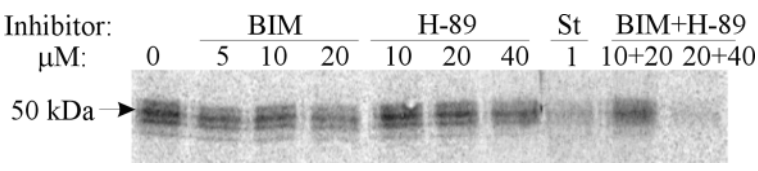

Fig. 10. COS-1 cells transfected with ΔExt/R4Tth were labeled with [^{33}P]-phosphate for 1 hour in the absence or presence of the protein kinase inhibitors BIM, H-89 or staurosporine (St) as indicated. Inhibitors were used at micromolar concentrations as indicated. The cells were solubilized and the receptor was immunoprecipitated with anti-FGFR4 antibodies and analysed by SDS-PAGE and autoradiography.

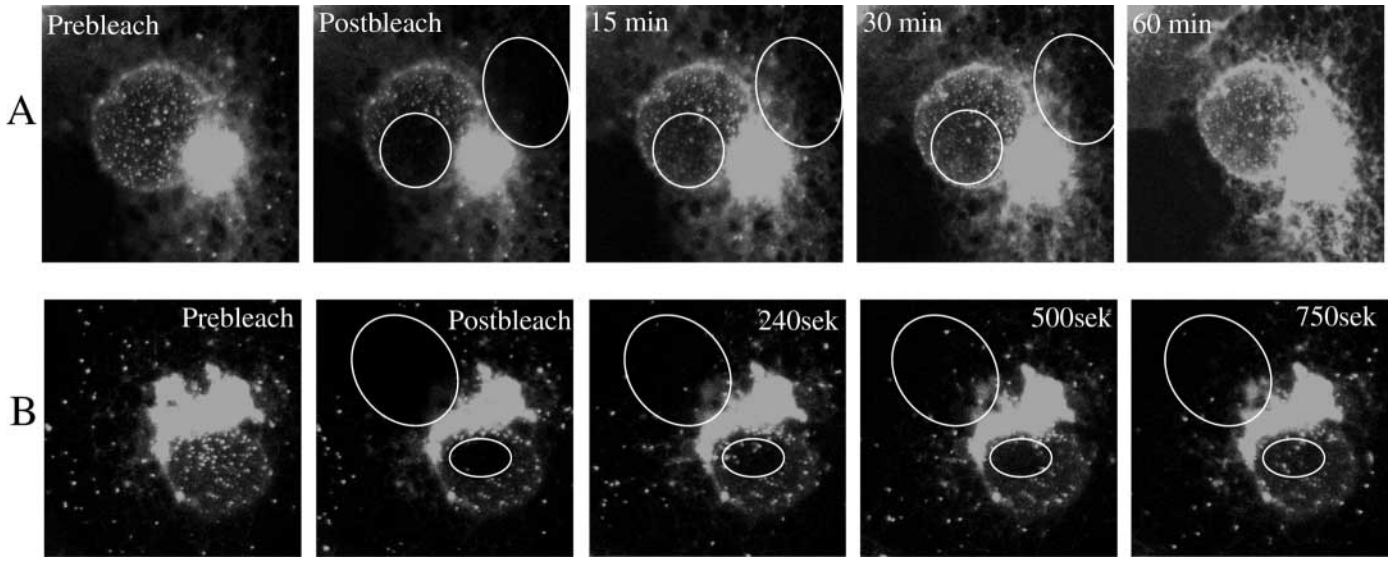

Fig. 11. COS-1 cells transfected with ΔExt-GFP/R4Tth were examined live by fluorescence microscopy. A region of the nucleus and a region of the cytoplasm was photobleached and the recovery of fluorescence was recorded by time-lapse confocal microscopy. (A,B) Two examples of recovery of nuclear spots after bleaching showing slow recovery of immobile nuclear spots (A) or faster movement of mobile spots from the juxtanuclear region into the nucleus (B).

stage), we observed unusual intranuclear onion-like membranous structures consisting of several layers of closely opposed membranes. These structures were devoid of nuclear pores but contained the ER markers PDI and calreticulin, in addition to abundant ΔExt/R4Tth.

Onion-like structures resembling those we describe here have already been found to be induced by the nuclear protein Nopp140 (Isaac et al., 2001). Nopp140 is a nuclear chaperone involved in ribosome biogenesis and it was found to induce intranuclear membranous structures named R-rings. This was first observed with an overexpressed deleted version of the protein but it was later found that the wild-type protein also induced such structures to some extent. These structures where characterized as folds of the INM; they lacked nuclear pores but possessed ER markers such as calnexin, BiP and PDI.

As discussed by Isaac et al. (Isaac et al., 2001), similar structures, called ‘nucleolar channel system’, have also been found to occur normally in the epithelium of the human endometrium at the time during the menstrual cycle when the fertilized egg is to be implanted (Terzakis, 1965). Owing to the similarity of the intranuclear structures observed by Isaac et al. and Terzakis, Isaac et al. suggested that Nopp140 might play a role in the process of blastocyst implantation, because Nopp140 was also found in intranuclear structures in sections of human endometrium. We have no indications that FGFR4 plays such a role, because we have not been able to observe the induction of the intranuclear structures by wild-type FGFR4.

We also observed that ΔExt/R4Tth induced membrane stacks in the cytosol. These had a slightly different appearance to those in the nucleus and consisted of more elongated stacks of membrane. Similar cytoplasmic stacks, originating from the ER, have previously been reported to occur in neurons with a natural high expression level of inositol-(1,4,5)-trisphosphate receptor, and in COS cells overexpressing inositol-(1,4,5)-trisphosphate receptor (Takei et al., 1994). There are also several reports of the formation of ER-derived membrane stacks in the cytosol induced by the overexpression of integral membrane proteins, including HMG-CoA reductase (Chin et al., 1982; Berciano et al., 2000), aldehyde dehydrogenase (Yamamoto et al., 1996) and cytochrome b_5 (Vergeres et al., 1993).

To our knowledge, only Nopp140 and ΔExt/R4Tth have so far been found to induce intranuclear membrane stacks consisting of several layers of membrane presumably derived from the INM or the ER. Overexpression of the lamin B receptor fused to green fluorescent protein (LBR-GFP) induced deep finger-like invaginations of the INM (Ellenberg et al., 1997), and overexpression of Nup153 induced intranuclear double-membrane lamellae with nuclear pores (Marelli et al., 2001). Others have also described tubular invagination of the NE (Fricker et al., 1997).

Although several different proteins can induce the formation of membrane stacks, this clearly does not occur for all overexpressed transmembrane proteins. As shown in this study, only one of several deletion mutants of FGFR4 induced intranuclear onion-like structures despite similar expression levels. The inability of R4wt to form intranuclear membrane stacks and the frequency with which ΔExt/R4Tth forms such structures were not altered by inhibiting the cellular degradation of the proteins, supporting the notion that overexpression and intracellular accumulation are not sufficient for the occurrence of onion-like structures. Special features of ΔExt/R4Tth that might contribute to its ability to induce intranuclear membrane stacks could include phosphorylation and the small size of the protein. The R4Tth mutant, which is identical to ΔExt/R4Tth in its transmembrane and intracellular region but has a full-length extracellular region, did not form intranuclear structures. Furthermore, when GFP was added to the C-terminus of ΔExt/R4Tth, the formation of the intranuclear structures was abolished.

Isaac et al. (Isaac et al., 2001) suggested that, for Nopp140,

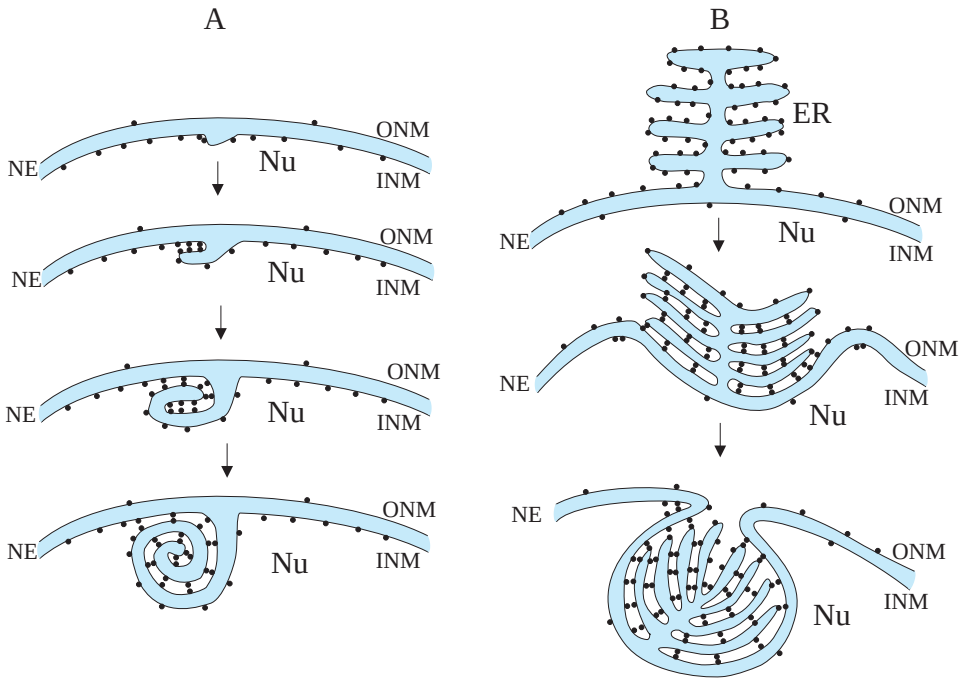

Fig. 12. Hypothetical models for formation of intranuclear membrane stacks. The cytoplasmic region of ΔExt/R4Tth (black dots) is assumed to mediate interactions between opposing membrane layers. (A) ΔExt/R4Tth localized to the INM induces invagination of the INM and further extension and folding of the invaginated membrane into whorled structures. (B) Interactions between cytoplasmic tails of ΔExt/R4Tth induces tight packing and adherence of ER membrane stacks and also interaction between ER and ONM. Interactions between ER and ONM and between opposing regions of the INM causes incorporation of the membrane stacks into the nucleus. Nu, nucleoplasm.

the folding of membrane into the R-rings could be related to the existence of charged regions in the protein that interact with the charged heads of phospholipids, thereby initiating membrane budding (Isaac et al., 2001). Nopp140 possesses a region that is rich in arginines and phosphorylated serines, and this was shown to be essential for the induction of R-rings. We found that the ΔExt/R4Tth protein, which has 27 serines in its intracellular part, was phosphorylated. The induction of the intranuclear structures by ΔExt/R4Tth was inhibited by long-term (overnight) incubation with BIM, an inhibitor of PKC. However, phosphorylation of ΔExt/R4Tth per se was not significantly inhibited by BIM and ongoing phosphorylation was found not to be required for the incorporation of the GFP-ΔExt/R4Tth fusion protein into nuclear spots when cells were studied by live microscopy. Thus, the role of phosphorylation in this process is not clear and phosphorylation of proteins other than ΔExt/R4Tth itself might be required for formation of the onion structures.

When GFP was fused into the extracellular region of ΔExt/R4Tth, the ΔExt-GFP/R4Tth molecule induced intranuclear structures similar (by confocal microscopy) to those of ΔExt/R4Tth, although at a lower frequency. Live microscopy of ΔExt-GFP/R4Tth showed that the intranuclear spots were stable, highly immobile structures. This suggests that the intranuclear structures contain deposits of the protein and this could represent a mechanism by which abnormal ER/INM-localized proteins can be sequestered.

From our experiments, it is not possible to make a final conclusion about how the intranuclear structures were formed. It is possible that the membranous onion-like structures were formed by invagination and folding of the INM, as was suggested for Nopp140 (Isaac et al., 2001), or that the membrane stacks/whorls were formed elsewhere (e.g. in the ER) and subsequently incorporated into the nucleus. Photobleaching experiments suggested that the intranuclear onion-like structures were formed at, or close to, the NE. We could observe recovery of nuclear spots after bleaching as both slow recovery of fluorescence in preexisting spots and rapid movement of spots from the juxtanuclear region into the nuclear region. Most cells were, however, not able to recover new ΔExt/R4Tth-protein in nuclear spots after photobleaching. A possible interpretation of these results is that cells are active in forming the onion-like structures only in a limited time window. During this time window, the structures might be attached to the ER/NE but, eventually, either the structures become detached from the ER/NE or the ΔExt/R4Tth protein within them becomes immobilized. The immobilization of well-developed onion-like structures in the nuclear matrix might have an inhibitory effect on the cells, possibly explaining why most cells do not recover fluorescent nuclear spots after photobleaching.

Self-self interactions between ΔExt/R4Tth molecules might be a driving force in the tight packing of membranes that is observed in the intranuclear and cytosolic membrane stacks induced by ΔExt/R4Tth. The C-terminal region of the ΔExt/R4Tth protein is the target for the immunostaining performed for EM. The staining appears to be mostly in the internal regions of the onion structures and less at their surface (Fig. 7), suggesting that the C-terminal cytoplasmic region of ΔExt/R4Tth mediates interactions between opposing membrane layers. Previously, head-to-head interactions between cytoplasmic regions of the inositol-(1,4,5)-trisphosphate receptor was proposed to mediate connections between adjacent ER cisternae and thereby induce formation of crystalloid ER structures (Takei et al., 1994).

In Fig. 12, two models are presented for how the intranuclear structures could be formed, assuming that interactions between cytoplasmic tails of ΔExt/R4Tth are the driving force. In model A, intranuclear structures are formed by invagination of the INM followed by further folding into spiral-like structures by interactions between opposing sheets of INM/INM-derived membrane. In model B, interactions between cytosolic regions

of ΔExt/R4Tth first lead to the formation of dense membrane stacks at the cytosolic side of the nuclear membranes (ER/ONM); these membrane stacks are subsequently incorporated into the nucleus owing to interactions between ER and ONM, and, possibly, opposing regions of INM. Both models require that nuclear pores are excluded from the invaginating part of the NE/INM.

Model A is consistent with some of our observations. The ΔExt/R4Tth protein is partially localized to the INM, and some of the intranuclear structures observed are spiral-like (Fig. 7C,D). However, several of our observations would be more consistent with a mechanism of formation as proposed in model B. The ΔExt/R4Tth protein is localized mostly to the ER, making it more probable that the ΔExt/R4Tth-ΔExt/R4Tth interactions that initiate the formation of intranuclear structures occur in the ER. Separate membrane stacks are also observed in the cytosol. Furthermore, invaginations of the NE containing several layers of membrane positively stained for ΔExt/R4Tth was observed (Fig. 9), as well as intranuclear membrane structures containing several membrane cisternae (Fig. 7E,F). The intranuclear onion-like membrane structures might therefore be formed by invagination of the NE to engulf the ΔExt/R4Tth-induced ER cisternal stacks.

After the submission of this manuscript, it was reported that high expression levels of ER-resident proteins with weak homodimeric interactions between their cytoplasmic domains could induce regular arrays of stacked ER membranes (Snapp et al., 2003). These membrane structures, termed organized smooth ER, had variable appearances and were found to form initially at sites adjacent to the NE. The mechanism of formation of the organized smooth ER structures described by Snapp et al. and the intranuclear onion-like membrane structures induced by ΔExt/R4Tth could have a similar basis, although a major difference is that the ΔExt/R4Tth structures are imported into the nucleus.

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References

Berciano, M. T., Fernandez, R., Pena, E., Calle, E., Villagra, N. T., Rodriguez-Rey, J. C. and Lafarga, M. (2000). Formation of intranuclear crystalloids and proliferation of the smooth endoplasmic reticulum in Schwann cells induced by tellurium treatment: association with overexpression of HMG CoA reductase and HMG CoA synthase mRNA. *Glia* **29**, 246-259.

Burgar, H. R., Burns, H. D., Elsden, J. L., Lalioti, M. D. and Heath, J. K. (2002). Association of the signaling adaptor FRS2 with fibroblast growth factor receptor 1 (FGFR1) is mediated by alternative splicing of the juxtamembrane domain. *J. Biol. Chem.* **277**, 4018-4023.

Burgess, W. H. and Maciag, T. (1989). The heparin-binding (fibroblast) growth factor family of proteins. *Annu. Rev. Biochem.* **58**, 575-606.

Chin, D. J., Luskey, K. L., Anderson, R. G., Faust, J. R., Goldstein, J. L. and Brown, M. S. (1982). Appearance of crystalloid endoplasmic reticulum in compactin-resistant Chinese hamster cells with a 500-fold increase in 3-hydroxy-3-methylglutaryl-coenzyme A reductase. *Proc. Natl. Acad. Sci. USA* **79**, 1185-1189.

Citores, L., Khnykin, D., Sorensen, V., Wesche, J., Klingenberg, O., Wiedlocha, A. and Olsnes, S. (2001). Modulation of intracellular transport of acidic fibroblast growth factor by mutations in the cytoplasmic receptor domain. *J. Cell Sci.* **114**, 1677-1689.

Citores, L., Wesche, J., Kolpakova, E. and Olsnes, S. (1999). Uptake and intracellular transport of acidic fibroblast growth factor: evidence for free and cytoskeleton-anchored fibroblast growth factor receptors. *Mol. Biol. Cell* **10**, 3835-3848.

Ellenberg, J., Siggia, E. D., Moreira, J. E., Smith, C. L., Presley, J. F., Worman, H. J. and Lippincott-Schwartz, J. (1997). Nuclear membrane dynamics and reassembly in living cells: targeting of an inner nuclear membrane protein in interphase and mitosis. *J. Cell Biol.* **138**, 1193-1206.

Ezzat, S., Zheng, L., Yu, S. and Asa, S. L. (2001). A soluble dominant negative fibroblast growth factor receptor 4 isoform in human MCF-7 breast cancer cells. *Biochem. Biophys. Res. Commun.* **287**, 60-65.

Fricker, M., Hollinshead, M., White, N. and Vaux, D. (1997). Interphase nuclei of many mammalian cell types contain deep, dynamic, tubular membrane-bound invaginations of the nuclear envelope. *J. Cell Biol.* **136**, 531-544.

Furukawa, K., Fritze, C. E. and Gerace, L. (1998). The major nuclear envelope targeting domain of LAP2 coincides with its lamin binding region but is distinct from its chromatin interaction domain. *J. Biol. Chem.* **273**, 4213-4219.

Furukawa, K., Pante, N., Aebi, U. and Gerace, L. (1995). Cloning of a cDNA for lamina-associated polypeptide 2 (LAP2) and identification of regions that specify targeting to the nuclear envelope. *EMBO J.* **14**, 1626-1636.

Georgatos, S. D. (2001). The inner nuclear membrane: simple, or very complex? *EMBO J.* **20**, 2989-2994.

Holaska, J. M., Wilson, K. L. and Mansharamani, M. (2002). The nuclear envelope, lamins and nuclear assembly. *Curr. Opin. Cell Biol.* **14**, 357-364.

Hughes, S. E. (1997). Differential expression of the fibroblast growth factor receptor (FGFR) multigene family in normal human adult tissues. *J. Histochem. Cytochem.* **45**, 1005-1019.

Imamura, T., Engleka, K., Zhan, X., Tokita, Y., Forough, R., Roeder, D., Jackson, A., Maier, J. A., Hla, T. and Maciag, T. (1990). Recovery of mitogenic activity of a growth factor mutant with a nuclear translocation sequence. *Science* **249**, 1567-1570.

Imamura, T., Oka, S., Tanahashi, T. and Okita, Y. (1994). Cell cycle-dependent nuclear localization of exogenously added fibroblast growth factor-1 in BALB/c 3T3 and human vascular endothelial cells. *Exp. Cell Res.* **215**, 363-372.

Isaac, C., Pollard, J. W. and Meier, U. T. (2001). Intranuclear endoplasmic reticulum induced by Nopp140 mimics the nucleolar channel system of human endometrium. *J. Cell Sci.* **114**, 4253-4264.

Kishi, T., Yoshida, T. and Terada, M. (1994). A soluble form of K-sam/FGFR2 protein in the culture medium of human gastric cancer cells. *Biochem. Biophys. Res. Commun.* **202**, 1387-1394.

Klingenberg, O., Wiedlocha, A., Rapak, A., Khnykin, D., Citores, L. and Olsnes, S. (2000). Requirement for C-terminal end of fibroblast growth factor receptor 4 in translocation of acidic fibroblast growth factor to cytosol and nucleus. *J. Cell Sci.* **113**, 1827-1838.

Klint, P. and Claesson-Welsh, L. (1999). Signal transduction by fibroblast growth factor receptors. *Front. Biosci.* **4**, D165-D177.

Malecki, J., Wiedlocha, A., Wesche, J. and Olsnes, S. (2002). Vesicle transmembrane potential is required for translocation to the cytosol of externally added FGF-1. *EMBO J.* **21**, 4480-4490.

Marelli, M., Lusk, C. P., Chan, H., Aitchison, J. D. and Wozniak, R. W. (2001). A link between the synthesis of nucleoporins and the biogenesis of the nuclear envelope. *J. Cell Biol.* **153**, 709-724.

Meier, U. T. (1996). Comparison of the rat nucleolar protein Nopp140 with its yeast homolog SRP40. Differential phosphorylation in vertebrates and yeast. *J. Biol. Chem.* **271**, 19376-19384.

Mohammadi, M., Honegger, A. M., Rotin, D., Fischer, R., Bellot, F., Li, W., Dionne, C. A., Jaye, M., Rubinstein, M. and Schlessinger, J. (1991). A tyrosine-phosphorylated carboxy-terminal peptide of the fibroblast growth factor receptor (Flg) is a binding site for the SH2 domain of phospholipase C-gamma 1. *Mol. Cell Biol.* **11**, 5068-5078.

Ong, S. H., Guy, G. R., Hadari, Y. R., Laks, S., Gotoh, N., Schlessinger, J. and Lax, I. (2000). FRS2 proteins recruit intracellular signaling pathways by binding to diverse targets on fibroblast growth factor and nerve growth factor receptors. *Mol. Cell Biol.* **20**, 979-989.

Ornitz, D. M. and Itoh, N. (2001). Fibroblast growth factors. *Genome Biol.* **2**, reviews3005.1-reviews3005.12.

Ostlund, C., Ellenberg, J., Hallberg, E., Lippincott-Schwartz, J. and Worman, H. J. (1999). Intracellular trafficking of emerin, the Emery-Dreifuss muscular dystrophy protein. *J. Cell Sci.* **112**, 1709-1719.

Powers, C. J., McLeskey, S. W. and Wellstein, A. (2000). Fibroblast

growth factors, their receptors and signaling. *Endocrinol. Relat. Cancer* **7**, 165-197.

Rolls, M. M., Stein, P. A., Taylor, S. S., Ha, E., McKeon, F. and Rapoport, T. A. (1999). A visual screen of a GFP-fusion library identifies a new type of nuclear envelope membrane protein. *J. Cell Biol.* **146**, 29-44.

Slot, J. W., Geuze, H. J., Gigengack, S., Lienhard, G. E. and James, D. E. (1991). Immuno-localization of the insulin regulatable glucose transporter in brown adipose tissue of the rat. *J. Cell Biol.* **113**, 123-135.

Smith, S. and Blobel, G. (1993). The first membrane spanning region of the lamin B receptor is sufficient for sorting to the inner nuclear membrane. *J. Cell Biol.* **120**, 631-637.

Snapp, E. L., Hegde, R. S., Francolini, M., Lombardo, F., Colombo, S., Pedrazzini, E., Borgese, N. and Lippincott-Schwartz, J. (2003). Formation of stacked ER cisternae by low affinity protein interactions. *J. Cell Biol.* **163**, 257-269.

Soullam, B. and Worman, H. J. (1993). The amino-terminal domain of the lamin B receptor is a nuclear envelope targeting signal. *J. Cell Biol.* **120**, 1093-1100.

Soullam, B. and Worman, H. J. (1995). Signals and structural features involved in integral membrane protein targeting to the inner nuclear membrane. *J. Cell Biol.* **130**, 15-27.

Takei, K., Mignery, G. A., Mugnaini, E., Sudhof, T. C. and de Camilli, P. (1994). Inositol 1,4,5-trisphosphate receptor causes formation of ER cisternal stacks in transfected fibroblasts and in cerebellar Purkinje cells. *Neuron* **12**, 327-342.

Terada, M., Shimizu, A., Sato, N., Miyakaze, S. I., Katayama, H. and Kurokawa-Seo, M. (2001). Fibroblast growth factor receptor 3 lacking the Ig IIIb and transmembrane domains secreted from human squamous cell carcinoma DJM-1 binds to FGFs. *Mol. Cell Biol. Res. Commun.* **4**, 365-373.

Terzakis, J. A. (1965). The nucleolar channel system of human endometrium. *J. Cell Biol.* **27**, 293-304.

Ueno, H., Gunn, M., Dell, K., Tseng, A., Jr and Williams, L. (1992). A truncated form of fibroblast growth factor receptor 1 inhibits signal transduction by multiple types of fibroblast growth factor receptor. *J. Biol. Chem.* **267**, 1470-1476.

van Heumen, W. R., Claxton, C. and Pickles, J. O. (1999). Fibroblast growth factor receptor-4 splice variants cause deletion of a critical tyrosine. *IUBMB Life* **48**, 73-78.

Vergeres, G., Yen, T. S., Aggeler, J., Lausier, J. and Waskell, L. (1993). A model system for studying membrane biogenesis. Overexpression of cytochrome b_5 in yeast results in marked proliferation of the intracellular membrane. *J. Cell Sci.* **106**, 249-259.

Wang, G. and Thomas, K. A. (1994). Purification and characterization of a functional soluble fibroblast growth factor receptor 1. *Biochem. Biophys. Res. Commun.* **203**, 1781-1788.

Wiedlocha, A., Falnes, P. O., Madshus, I. H., Sandvig, K. and Olsnes, S. (1994). Dual mode of signal transduction by externally added acidic fibroblast growth factor. *Cell* **76**, 1039-1051.

Wiedlocha, A., Falnes, P. O., Rapak, A., Klingenberg, O., Munoz, R. and Olsnes, S. (1995). Translocation of cytosol of exogenous, CAAX-tagged acidic fibroblast growth factor. *J. Biol. Chem.* **270**, 30680-30685.

Wiedlocha, A., Falnes, P. O., Rapak, A., Munoz, R., Klingenberg, O. and Olsnes, S. (1996). Stimulation of proliferation of a human osteosarcoma cell line by exogenous acidic fibroblast growth factor requires both activation of receptor tyrosine kinase and growth factor internalization. *Mol. Cell Biol.* **16**, 270-280.

Wu, W., Lin, F. and Worman, H. J. (2002). Intracellular trafficking of MAN1, an integral protein of the nuclear envelope inner membrane. *J. Cell Sci.* **115**, 1361-1371.

Yamamoto, A., Masaki, R. and Tashiro, Y. (1996). Formation of crystalloid endoplasmic reticulum in COS cells upon overexpression of microsomal aldehyde dehydrogenase by cDNA transfection. *J. Cell Sci.* **109**, 1727-1738.

Yan, G., McBride, G. and McKeehan, W. L. (1993). Exon skipping causes alteration of the COOH-terminus and deletion of the phospholipase C gamma 1 interaction site in the FGF receptor 2 kinase in normal prostate epithelial cells. *Biochem. Biophys. Res. Commun.* **194**, 512-518.

Zhan, X., Hu, X., Friedman, S. and Maciag, T. (1992). Analysis of endogenous and exogenous nuclear translocation of fibroblast growth factor-1 in NIH 3T3 cells. *Biochem. Biophys. Res. Commun.* **188**, 982-991.

Zhan, X., Hu, X., Friesel, R. and Maciag, T. (1993). Long term growth factor exposure and differential tyrosine phosphorylation are required for DNA synthesis in BALB/c 3T3 cells. *J. Biol. Chem.* **268**, 9611-9620.