Factors Controlling Fibroblast Growth Factor Receptor-1's Cytoplasmic Trafficking and Its Regulation as Revealed by FRAP Analysis

Star M. Dunham-Ems,^{*†} Haridas E. Pudavar,[†] Jason M. Myers,^{*} Pamela A. Maher,[‡] Paras N. Prasad,[†] and Michal K. Stachowiak^{*†}

^{*}Molecular and Structural Neurobiology and Gene Therapy Program, Department of Pathology and Anatomical Sciences, State University of New York at Buffalo, Buffalo, NY 14214; [†]Institute for Lasers, Photonics, and BioPhotonics, Department of Chemistry, State University of New York at Buffalo, Buffalo NY 14260; and [‡]The Salk Institute, La Jolla, CA 92037

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Biochemical and microscopic studies have indicated that FGFR1 is a transmembrane and soluble protein present in the cytosol and nucleus. How FGFR1 enters the cytosol and subsequently the nucleus to control cell development and associated gene activities has become a compelling question. Analyses of protein synthesis, cytoplasmic subcompartmental distribution and movement of FGFR1-EGFP and FGFR1 mutants showed that FGFR1 exists as three separate populations (a) a newly synthesized, highly mobile, nonglycosylated, cytosolic receptor that is depleted by brefeldin A and resides outside the ER-Golgi lumen, (b) a slowly diffusing membrane receptor population, and (c) an immobile membrane pool increased by brefeldin A. RSK1 increases the highly mobile cytosolic FGFR1 population and its overall diffusion rate leading to increased FGFR1 nuclear accumulation, which coaccumulates with RSK1. A model is proposed in which newly synthesized FGFR1 can enter the (a) "nuclear pathway," where the nonglycosylated receptor is extruded from the pre-Golgi producing highly mobile cytosolic receptor molecules that rapidly accumulate in the nucleus or (b) "membrane pathway," in which FGFR1 is processed through the Golgi, where its movement is spatially restricted to *trans*-Golgi membranes with limited lateral mobility. Entrance into the nuclear pathway is favored by FGFR1's interaction with kinase active RSK1.

INTRODUCTION

All signaling events in a cell occur due to protein movement. Once protein-protein contact is initiated (due to random motion) a variety of reactions can occur, such as phosphorylation, methylation, or acetylation (Papin *et al.*, 2005). Membrane-associated growth factor receptors play a pivotal role in signal transduction, transmitting signals from the extracellular environment to the cytoplasm, according to the classical theory of signal transduction. Other cytoplasmic proteins and kinases serve as secondary messengers and propagate the signal downstream to the nucleus. There are several growth factor receptors that have alternative pathways to propagate signals and affect gene activities and cell development by acting within

the nucleus, including fibroblast growth factor receptor-1 (FGFR1), fibroblast growth factor receptor-2 (FGFR2), epidermal growth factor receptor (erb1,-2, -3), and the prolactin receptor (Stachowiak *et al.*, 1997, 2003b; Wells and Marti, 2002; Carpenter, 2003; Hu *et al.*, 2004; Schmahl *et al.*, 2004; Sabbieti *et al.*, 2005). FGFR1 nuclear accumulation has been observed in vivo in developing neuronal and glial cells (Gonzalez *et al.*, 1995; Clarke *et al.*, 2001; Stachowiak *et al.*, 2003a; Fang *et al.*, 2005) and has been shown to affect cell development (Bharali *et al.*, 2005; Fang *et al.*, 2005). FGFR1 nuclear accumulation has also been observed in vitro in cultured astrocytes, adrenal medullary cells, neural progenitor cells, neurons, and in medulloblastoma, glioma, and neuroblastoma cancer cells (Maher, 1996; Stachowiak *et al.*, 1997, 2003a, 2003b; Peng *et al.*, 2002). FGFR1 nuclear accumulation occurs in association with glial growth, dendritic outgrowth in rat neurons, differentiation of human and rat neuronal progenitor cells, and functional adaptations in adrenal medullary cells and has also been shown to play essential roles in all of these processes (Stachowiak *et al.*, 2003b).

Because FGFR1 is destined for constitutive fusion with the plasma membrane due to its signal peptide (SP), how the receptor may enter the cytosol and undergo subsequent nuclear accumulation has become a compelling question. In epithelial cells, ligand-induced FGFR1 internalization coincides with and is modulated by the internalization of E-cadherin and is followed by FGFR1 nuclear accumulation (Bryant *et al.*, 2005). It remains to be determined if the nuclear FGFR1 that accumulates under those conditions rep-

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Address correspondence to: M. K. Stachowiak (mks4@buffalo.edu).

Abbreviations used: AII, angiotensin II; BAMC, bovine adrenal medullary cells; BFA, brefeldin A; BMP7, bone morphogenetic protein-7; EGFR1, epidermal growth factor receptor 1; ER, endoplasmic reticulum; FGF, fibroblast growth factor; FGFR1, FGF receptor-1; FRAP, fluorescent recovery after photobleaching; GFP, green fluorescent protein; EGFP, enhanced GFP; SP, signal peptide; NLS, nuclear localization signal; PI3K, phosphoinositol 3-kinase; RSK1, p90 ribosomal S6 kinase-1; SP, signal peptide; SRP, signal-recognition particle; TK, tyrosine kinase; TMD transmembrane domain.

resents newly synthesized or the internalized cell surface receptor. Incubation of glioma or TE671 medulloblastoma cells with NHS-sulfobiotin does not lead to the appearance of a biotinylated receptor in the nucleus (Stachowiak *et al.*, 1997; Peng *et al.*, 2002), indicating that in these cells nuclear FGFR1 is not derived from the cell surface. Binding of extracellular FGF to its surface receptors does not elicit nuclear FGFR1 accumulation in many cells (Stachowiak *et al.*, 1994a, 1996a, 1996b; Moffett *et al.*, 1996, 1998a; Peng *et al.*, 2001, 2002). In contrast, FGFR1 nuclear accumulation affecting cell growth and functions is elicited by changes in cell contact, growth factors (BMP7), hormonal (angiotensin II [AngII]), neurotransmitter receptors (acetylcholine [ACh]), cell depolarization and stimulation of cAMP and Ca^{2+} /protein kinase C (PKC) signaling and is accompanied by an increase in receptor levels (Stachowiak *et al.*, 1994a, 1994b, 1996a, 1996b, 1997, 2003a; Moffett *et al.*, 1996, 1998a, 1998b; Peng *et al.*, 2001, 2002; Horbinski *et al.*, 2002). These results suggested that newly synthesized FGFR1 may engage in a unique transport process directed by the receptor's different domains that allow both insertion into the plasma membrane and intranuclear accumulation (Peng *et al.*, 2001, 2002; Carpenter, 2003; Myers *et al.*, 2003; Hu *et al.*, 2004).

FGFR1 contains a N-terminal hydrophobic SP (amino acids 2–20; Hou *et al.*, 1991) generally known to bind a signal-recognition particle (SRP), as it emerges from the ribosome (Walter and Johnson, 1994; Stroud and Walter, 1999). The SRP-ribosome associates with the cytosolic surface of the rough ER and the polypeptide elongates and translocates into the ER lumen via an aqueous pore. On reaching the hydrophobic transmembrane domain (TMD), the translated protein dissociates from the translocase and the hydrophobic TMD becomes anchored in the lipid bilayer (Walter and Johnson, 1994; Stroud and Walter, 1999). When the SP is cleaved by the signal peptidase, the result is a TM protein with its N-terminal in the ER lumen and its C-terminal in the cytosol. Typically, the TMD contains an α -helix of ~30 amino acids, with more than 11 consecutive hydrophobic amino acids. These nonpolar amino acids are oriented outside the α -helical core and interact with the lipid bilayer, thereby conferring stability to the peptide in the membrane (Creighton, 1984). Disruption of this hydrophobic region by polar amino acids makes membrane association less stable (Eisenberg *et al.*, 1984). The atypical TMD of FGFR1 contains short stretches of hydrophobic amino acids interrupted by hydrophilic, polar amino acids. The predicted conformation of the FGFR1 TMD is a β -sheet, which is in contrast to the α -helical conformation of other single TM tyrosine kinase (TK) receptors (including FGFR4). Chimeric FGFR1/R4-EGFP, where the TMD of FGFR1 was replaced with that of FGFR4, was primarily associated with membranes (ER Golgi, plasma, and nuclear) and absent from the cytosol and nucleus (Myers *et al.*, 2003). In contrast, FGFR1(Δ 1–3)-EGFP mutants, with hydrophobic TMD amino acids replaced with hydrophilic amino acids, showed reduced association with membranes and increased cytosolic/nuclear accumulation with increasing TMD hydrophilicity. Deletion of the SP-encoding sequence [FGFR1(SP⁻)] resulted in cytosolic, nonmembrane accumulation. Thus, the unstable association with cellular membranes could be responsible for FGFR1 accumulation in the cytosol and cytosolic FGFR1 constitutes the source of the nuclear receptor (Myers *et al.*, 2003). This mechanism of FGFR1 release into the cytosol may be analogous to the known retrotranslocation of other ER transmembrane proteins back to the cytosol by the Sec61 complex (Myers *et al.*, 2003). This model of FGFR1 processing was based on cell fractionation studies, immunocytochemistry of fixed cells, and on confocal analysis of steady state levels of FGFR1-EGFP in subcellular compartments of live

cells. Whether FGFR1 nuclear localization involves nascent FGFR1 chains or redirection of FGFR1 from preexisting pools was unknown.

An important insight into protein trafficking mechanisms can be obtained by measuring real-time protein movement and residence times in subcellular microlocales. One technique that assesses these processes in cells is fluorescent recovery after photobleaching (FRAP). FRAP provides quantitative information about the diffusion rate and mobile fraction of fluorescent molecules and allows determination of the protein flux within and between intracellular compartments (Misteli, 2001). FRAP has been routinely used to study the environmental dependence of protein mobility, such as the lateral diffusion of membrane-associated proteins (Livneh *et al.*, 1986), protein distribution (Partikian *et al.*, 1998; Phair and Misteli, 2000), and the dynamics and interactions with other cellular components in living cells (Misteli, 2001; Catez *et al.*, 2002; Liebman and Entine, 1974; Schlessinger *et al.*, 1976; Edidin *et al.*, 1994). These studies have indicated that proteins are in continuous flux between different molecular assembly states and that this flux determines the protein's fate and function.

The present study uses FRAP to advance the understanding of FGFR1 cytoplasmic trafficking via analysis of its mobility and diffusion. Specifically, we examined how FGFR1's associations with different cytoplasmic subcompartments affects receptor mobility and tested the hypothesis that non-membrane, cytosolic FGFR1 may have the ability to rapidly move in the cytoplasm and allow its nuclear translocation. Our results indicate that FGFR1's domains and protein interactions, namely with p90 ribosomal S6 kinase (RSK1), influence its cytoplasmic mobility and subsequent nuclear accumulation in live cells, a process that is essential for cell development and associated gene activities (Stachowiak *et al.*, 2003b; Fang *et al.*, 2005).

MATERIALS AND METHODS

Plasmid Constructs

pcDNA3.1FGFR1 expressing full-length FGFR1 was described in (Stachowiak *et al.*, 1997). FGFR1(TK⁻), FGFR1(SP⁻), FGFR1(SP⁻/nuclear localizing signal [NLS]), FGFR1(Δ 2) were previously described (Peng *et al.*, 2002). All FGFR1-pEGFP-N2 constructs were previously described (Myers *et al.*, 2003). Briefly, the pcDNA3.1-FGFR1 vector was constructed (Peng *et al.*, 2001) by cloning the entire coding sequence of human FGFR1 (Hou *et al.*, 1991) into the XbaI site of the pcDNA 3.1 plasmid (Invitrogen, Carlsbad, CA). pcDNA3.1/Myc/HisB-FGFR1 was constructed by PmeI and HindIII fragment of pcDNA3.1 FGFR1 into pcDNA3.1myc/HisB cut with EcoRV and HindIII. pcDNA3.1-FGFR1(Δ 1–3)-Myc/His was made so that it incorporated one mutation (V391) in FGFR1(Δ 1), two mutations (V391R and C381R) in FGFR1(Δ 2), and three mutations (V391R, C381R, and L386) in FGFR1(Δ 3). pcDNA3.1. FGFR1(SP⁻) was constructed by deleting the SP (a.a. 3–16). FGFR1 mutants were subcloned into pEGFP-N2 (Clontech, Palo Alto, CA) from pcDNA3.1FGFR1-Myc/HisB. All clones were verified by DNA sequencing and Western blotting to confirm correct protein size. pRSK1/flag was previously described (Hu *et al.*, 2004). UB6 GalCBP was obtained from Dr. R. H. Goodman (Oregon Health Science University). RSK mutant [pKH3RSK1(K112/464R)] was a gift from Dr. J. Blenis (Harvard University).

Cell Culture and Transfection

Bovine adrenal medullary cells (BAMC) were isolated and the primary cultures prepared as previously described by Stachowiak *et al.* (1994a). Human TE671 medulloblastoma cells (express low levels of endogenous FGFR1) were grown in DMEM (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen) and maintained at 37°C with 5% CO₂ in a humidified incubator. Cells were kept in serum-free medium 24 h before transfection until the end of the experiment.

TE671 cells were plated 1 d before transfection on glass coverslips. Seventy to 80% confluent TE671 were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol for microscopy. For cell harvesting and lysis, cells were transfected using calcium chloride with a total DNA concentration equaling 10 μ g of pcDNA3.1-FGFR1, pcDNA3.1-FGFR1(Δ 2), FGFR1(TK⁻), FGFR1(SP⁻), FGFR1(SP⁻/NLS), or control pcDNA3.1 vectors. After 36 h, cells

were harvested and lysed in lysis buffer. For microscopy, cells were transfected with total DNA concentration equaling 1 μ g of FGFR1-EGFP, FGFR1(TK⁻)-EGFP, FGFR1(SP⁻)-EGFP, FGFR1(SP⁻/NLS)-EGFP, FGFR1(Δ 2)-EGFP, FGFR1-EGFP + RSK1, FGFR1-EGFP + RSK1mt, FGFR1-EGFP + CBP per coverslip. Cells were analyzed via confocal microscopy and FRAP 24–30 h after transfection. For drug treatment 24 h after transfection the medium of TE671 cells was supplemented with 3 μ M lactacystin (Calbiochem, La Jolla, CA) for 4 h or with 10 μ g/ml brefeldin A (BFA; Sigma-Aldrich, St. Louis, MO) for 1 h or 100 mg/ml cycloheximide (Sigma-Aldrich) for 4 h before analysis.

Cell Lysis, Fractionation, Immunoprecipitation, and Western Immunoblotting

Nuclear and cytoplasmic fractions were isolated and characterized as previously described (Stachowiak *et al.*, 1996a, 1996b; Peng *et al.*, 2001, 2002). This method produces nuclei in which potential contamination with plasma membrane FGFR1 is below the detection limit as shown by the absence of biotinylated plasma membrane FGFR1 in the nuclear fraction (Peng *et al.*, 2002) and by a number of other techniques (Maher, 1996; Stachowiak *et al.*, 1996a, 1996b). Protein content was determined using the Bio-Rad assay (Richmond, CA). Immunoprecipitation was performed after lysis as described in Hu *et al.* (2004) using polyclonal FGFR1 C-terminal antibody (Stratagene, La Jolla, CA), GFP antibody (Clontech), or with control anti-HA (Sigma-Aldrich) or anti-RNA Polymerase II (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. Proteins were resolved on 4–8% SDS-polyacrylamide gels. In each experiment, equal protein loading was confirmed by silver (Pierce Biotechnology, Rockford, IL) and/or Ponceau S (Sigma-Aldrich) staining. Immunoblotting was then performed with the following antibodies: McAb6 against FGFR1 N-terminal immunoglobulin (Ig)-like loops (Hanneken *et al.*, 1995), GFP (Clontech), and RSK1 (Santa Cruz Biotechnology).

Isolation of Membrane and Cytosolic Fractions

Cells were harvested in 1 ml of 20 mM Tris-HCl, pH 8.0, buffer containing 2 μ M phenylmethylsulfonyl fluoride, 10 μ g/ml benzamide, 1 mM EDTA, and 1 mM EGTA, collected in a 2-ml glass Dounce homogenizer and left on ice for 15 min to swell. Cells were then sheared 30–40 times with a tight fitting Teflon pestle. The homogenates were combined with 1 ml of 1 M sucrose in 20 mM Tris-HCl, pH 8.0, buffer, mixed, and spun at 1600 $\times$ g for 10 min to remove nuclei. The cytoplasmic fraction was collected above the sucrose cushion cleared by centrifugation at 2500 $\times$ g for 5 min to pellet unbroken cells and contaminating nuclei. The obtained cytoplasm was spun at 150,000 $\times$ g for 30 min in an Optima TLX ultracentrifuge (Beckman, Palo Alto, CA) in a TLS-55 swinging bucket rotor. The supernatant (S150) was concentrated in an Ultrafree-4 Centrifugal filter and Tube with a BIOMAX-30K NMWL Membrane (Millipore, Bedford, MA). The generated pellet (P150, membrane microsomal fraction) was washed by resuspending in harvest solution and spun at 150,000 $\times$ g for 30 min. The pellet was resuspended in sample buffer. Denatured proteins were resolved on 7.5% SDS-Page gel and immunoblotted with the following antibodies: McAb6 (Hanneken *et al.*, 1995), GFP (Clontech), and RSK1 (Santa Cruz Biotechnology). Chemiluminescent detection was performed using manufacturer's protocol (Pierce Biotechnology). The nuclear fraction isolated using this method contains <5% of the total cellular activity of 5' nucleotidase (plasma membrane marker), <2% of the total activity of acid phosphatase (lysosomal marker), and no detectable levels of β -tubulin (Santa Cruz Biotechnology). Nuclear contamination was not detected in the S150 and P150 fractions as determined by the absence of endogenous CBP. Lack of S150 and P150 cross-contamination was shown by expression of nonmembrane/cytosolic FGFR1(SP⁻) mutant and soluble nonglycosylated w.t. FGFR1 only in the S150 fraction and by expression of membrane-associated glycosylated forms of w.t. FGFR1 only in the P150 fraction. Also high levels of actin were detected in S150 and only trace amounts in P150.

Immunocytochemistry

BAMC were fixed and stained using a monoclonal N-term FGFR1 McAb6 (Hanneken *et al.*, 1995) that recognizes different glycosylated forms of FGFR1, with sheep polyclonal anti-phospho-Ser(364) RSK1 antibody (Upstate Biotechnology, Lake Placid, NY), as described previously (Hu *et al.*, 2004). Fluorescent staining was performed using Alexa 488-conjugated secondary antibodies. Specificity of immunostaining was ascertained with control reactions in which the primary antibody was omitted or replaced with preimmune sera or by neutralizing the antibody with cognate peptide described previously (Stachowiak *et al.*, 1996a; Hu *et al.*, 2004). Cells were analyzed by confocal microscopy.

Confocal Microscopy and Image Analysis

Coverslips with live cells were mounted with MEM containing 5% FBS and 1% Penn/Strep at RT and sealed with VALAP (1:1:1; low melting point paraffin, Vaseline, and lanolin). Confocal images of live transfected cells were acquired using a Bio-Rad MRC 1024 confocal microscope (Richmond, CA) equipped with a 1 W Argon laser, operating on a Nikon Optiphot upright microscope (Melville, NY) with an oil immersion 60 $\times$, 1.4 NA objective. Optical sections were acquired at 0.5- μ m steps and the XY pixel size was set at $\geq$ 0.20 μ m using Bio-Rad's

Lasersharp V3.0 software and later processed using Todd Brelje's Confocal Assistant v4.02.

FRAP

The description of image acquisition, instrumentation, and methodology has been previously described (Dunham *et al.*, 2004). Briefly, the excitation light was provided by a 488-nm line from an Argon ion laser. A galvanometer mirror (GSI Lumonics) was used to switch the laser beam into a low-intensity imaging beam and high-intensity bleaching beam. Both beam paths were combined by a beam splitter (30/70 splitter) and coupled into a single mode fiber for delivery into the confocal microscope (Bio-Rad MRC-1024). Bio-Rad time-course software was used for FRAP experiments. In a typical experiment, after a photobleaching pulse of 1–2 s, images (256 $\times$ 256) were acquired at 750-ms intervals. The setup used was also modified to obtain localized spectra from different compartments of the cell being imaged, which has been previously described (Pudavar *et al.*, 2000). Localized emission spectra from transfected cells were routinely acquired to confirm that there was no significant autofluorescence and the emission observed was indeed from EGFP molecules. This setup was extensively tested with EGFP-transfected live and fixed cells.

FRAP Data Analysis

The acquired images were analyzed using ImageJ imaging software (NIH). For each analysis a minimum of three cells were chosen. Three regions were chosen within each compartment and then averaged across cells to estimate the compartment behavior within the bleach region. Regions chose were of the same size (10 $\times$ 7 pixels per region of interest). Data were corrected for background intensity and for the overall loss in total intensity as a result of the bleach pulse and the imaging scans. Recovery measurements ($n \geq 6$) were quantified by fitting normalized fluorescence intensities of bleached areas to a one-phase exponential association by using the one exponential nonlinear regression algorithm of Prism 4 (GraphPad Software, San Diego, CA); this program was also used for plotting of the data and statistical analysis. Parameters are reported as mean $\pm$ SEM. For the two exponential nonlinear regression analysis, the regions within FGFR1-EGFP-expressing cells were processed exactly the same as for the one exponential association, except fitting was performed using the two-exponential nonlinear regression algorithm of Prism 4 (GraphPad Software). ANOVA and Bartlett tests were applied to analyze differences among diffusion rates ($t_{1/2}$), populations of mobile FGFR1-EGFP and its mutants and the effects of cotransfected proteins.

Pulse-Chase Analysis

TE671 cells were transfected with pcDNA3.1, FGFR1, or FGFR1-EGFP. Twenty-four hours later the cells were rinsed three times with cysteine/methionine-free DMEM and labeled for 1 h with 50 μ M/ml Redivue PRO-MIX (Amersham, Piscataway, NJ, 1000 Ci/mmol) in cysteine/methionine-free DMEM containing 10% dialyzed fetal calf serum (FCS). The cells were rinsed three times with DMEM and then either processed immediately (pulse, vector) or incubated for 30 min to 2 h in DMEM containing 10% FCS before processing. The samples were then collected and subjected to immunoprecipitation (as described above) with a FGFR1 (Santa Cruz Biotechnology) or GFP (Promega, Madison, WI) antibody and analyzed by SDS-PAGE on 10% gels. The gels were fixed, infiltrated with Enhance (Perkin Elmer-Cetus, Wellesley, MA), dried and exposed to film for 1–5 d at -70° C.

RESULTS

Expression of Full-Length FGFR1-EGFP Fusion Proteins in TE671 Cells

For our current experiments, we have used previously generated and characterized fusions of FGFR1 and its mutants with the EGFP protein. These transfected chimeric proteins showed the same subcellular distributions and functions in cell differentiation and gene regulation as the nonfused receptors and mimicked the specific aspects of endogenous FGFR1 biology (Peng *et al.*, 2001, 2002; Myers *et al.*, 2003; Hu *et al.*, 2004).

First, expression of FGFR1-EGFP and FGFR1-EGFP mutants were confirmed by Western blot analysis with FGFR1 McAb6 in transfected TE671 cells (Figure 1A). TE671 medulloblastoma cells express endogenous FGFR1 at levels often below the detection limit of direct FGFR1 antibody immunoblotting. TE671 cells express recombinant FGFR1 at levels that are similar to endogenous FGFR1 in many neural cell types when transfected with FGFR1-expressing plasmids. As found previously (Myers *et al.*, 2003), 40 h after transfection, FGFR1-EGFP

Figure 1. Western immunoblot analysis of FGFR1-EGFP fusion proteins in total cell lysates. FGFR1-EGFP and controls were transfected into TE671 cells. Nonglycosylated (NG) and glycosylated receptor forms (G) are indicated. (A) Immunoblotting with FGFR1 McAb6. The lanes correspond to the following transfected plasmids: (1) pcDNA, (2) FGFR1-EGFP, (3) FGFR1(TM⁻)-EGFP, (4) FGFR1(SP⁻)-EGFP, (5) FGFR1(Δ2)-EGFP, (6) FGFR1(Δ3)-EGFP, (7) pcDNA, (8) FGFR1(TK⁻)-EGFP. FGFR1-EGFP predominantly migrated as two hyperglycosylated forms (150 and 160 kDa) and trace amounts of the nonglycosylated (120-kDa) form. FGFR1(Δ2)-EGFP, FGFR1(Δ3)-EGFP, and FGFR1(TM⁻)-EGFP all migrated at 120 and 150 kDa. FGFR1(SP⁻)-EGFP ran as a single band around 120 kDa. (B) Immunoblotting with anti-EGFP. The lanes correspond to the following transfected plasmids: (1) FGFR1(Δ2)-EGFP, (2) FGFR1(Δ3)-EGFP, (3) FGFR1(SP⁻)-EGFP, (4) FGFR1(TK⁻)-EGFP, (5) FGFR1(TM⁻)-EGFP, (6) FGFR1-EGFP, (7) pcDNA, (8) EGFP. EGFP migrates at 27 kDa. The EGFP antibody did not detect bands that would indicate expression of EGFP alone or fragments of FGFR1-EGFP proteins from transfected recombinant plasmids.

migrated as two predominant 160- and 150-kDa forms, consistent with the combined molecular weight of hyperglycosylated (135 kDa) and partially glycosylated (120 kDa) forms of FGFR1 and EGFP (27 kDa). Their size was found to be reduced by treatment with N-glycanase to ~120 kDa (Myers *et al.*, 2003). Nonglycosylated FGFR1-EGFP (120 kDa) was the least abundant isoform (Figure 1A, lane 2). FGFR1(Δ2)-EGFP (Figure 1A, lane 5) and FGFR1(Δ3)-EGFP (lane 6), with point-mutated TMDs, comigrated with wild-type (w.t.) FGFR1 (Figure 1A, lane 2) at 135 and 150 kDa. No hyperglycosylated (160 kDa) forms of FGFR1(Δ2)-EGFP and FGFR1(Δ3)-EGFP were detected, whereas the amount of the nonglycosylated isoform (125 kDa) increased relative to FGFR1-EGFP. Also, FGFR1(TM⁻)-EGFP (Figure 1A, lane 3), which lacks the TMD, was expressed equally in the nonglycosylated and partially glycosylated forms. FGFR1(SP⁻)-EGFP is translated by free ribosomes and thus becomes a resident cytosolic protein without initial insertion into the ER membrane and does not become glycosylated in the absence of a SP. As predicted, FGFR1(SP⁻)-EGFP migrated as a single band of 120 kDa, consistent with the size of a nonglycosylated fusion protein (Figure 1A, lane 4). Because the SP is normally cleaved during membrane insertion, the size of FGFR1(SP⁻) is the same as nascent (nonglycosylated) FGFR1. FGFR1(TK⁻)-EGFP that lacks the TK domain migrated as 75-kDa nonglycosylated and 90–100-kDa glycosylated forms (Figure 1, A, lane 8, and B, lane 4).

Next we ascertained whether the transfected EGFP fusion proteins were expressed as EGFP alone (27 kDa) or fragments of FGFR1-EGFP proteins that cannot be detected with

McAb6. The extracts of cells expressing the FGFR1 fusion proteins were immunoblotted with a EGFP antibody and found to contain no additional lower molecular weight bands that would indicate expression of EGFP alone (27 kDa; Figure 1B, lane 8) or fragments of FGFR1-EGFP proteins from the transfected recombinant plasmids (Figure 1B, lanes 1–6). Thus, FGFR1-EGFP and its mutants were expressed only as fusion proteins.

Trafficking of FGFR1-EGFP within Cytoplasmic Subcompartments in TE671 Cells

Earlier cytological studies showed that newly synthesized endogenous FGFR1 or recombinant FGFR1-EGFP accumulated initially in ER-Golgi cytoplasmic membranes followed by accumulation in the cytosol and nuclear compartments of human glioma cells (Myers *et al.*, 2003). FGFR1 TMD mutations that increased or reduced FGFR1 ER-Golgi retention had opposite effects on FGFR1 cytosolic accumulation, suggesting that soluble, cytosolic FGFR1 is released from cytoplasmic membranes to subsequently accumulate in the nucleus (Myers *et al.*, 2003). To verify this FGFR1-EGFP processing in TE671 cells and to identify receptor isoforms in specific subcellular compartments, we transfected TE671 cells with FGFR1-EGFP and analyzed the cytosolic (S150), microsomal (P150), and nuclear (N) fractions with FGFR1 McAb6 (Figure 2). FGFR1-EGFP was not detected in cells transfected with control pEGFP (unpublished data) or FGFR1-EGFP 8 h after transfection. Nonglycosylated FGFR1-EGFP (120 kDa) did not reach measurable levels in the cytosolic fraction after 12 h (traces of 120-kDa FGFR1-EGFP were detected in some experiments; unpublished data), but became clearly detectable at 20 h, and 120-kDa FGFR1-EGFP was visible at 12 h in the nucleus and increased further at 20 h. An intermediate glycosylated form of FGFR1-EGFP (150 kDa) was observed in the P150 fraction at 12 h and increased slightly during the subsequent 8 h. At that time the hyperglycosylated form of FGFR1-EGFP (160 kDa) was also detected. In addition, at 20 h the nuclear fraction also contained the 150–160-kDa bands that may represent nuclear membrane associated FGFR1-EGFP, which is continuous with the ER.

To verify that the nonglycosylated FGFR1-EGFP isoform in the S150 fraction represented the soluble receptor, we examined the distribution of transfected soluble FGFR1(SP⁻)-EGFP (SP-encoding sequence deleted). Nonglycosylated FGFR1(SP⁻)-EGFP (120 kDa) was detected in the S150 fraction, but not in the P150 fraction (Figure 2A).

In earlier studies we had shown that 95 kDa as well as slower migrating forms of endogenous FGFR1 in BAMC and in human astrocytes represent the full-length noncleaved receptor (Stachowiak *et al.*, 1996a; 1996b). In the present study we confirmed that transfected w.t. soluble FGFR1 (95 kDa) immunoprecipitated with a FGFR1 C-term antibody and that FGFR1-EGFP (120 kDa) immunoprecipitated with an antibody against the C-terminal EGFP tag can both be detected by an antibody (McAb6) against the FGFR1 N-terminal Ig loops (Figure 2B). Thus 95-kDa FGFR1 and 120-kDa FGFR1-EGFP represent noncleaved receptors. Additionally, cell treatment with the proteosome inhibitor lactostatin produced a small increase rather than depleted the 120-kDa FGFR1-EGFP. These results further demonstrate that the soluble receptor is not produced by proteolytic processing from its larger forms (Figure 2C). In contrast, short-term treatment (4 h) with cycloheximide prevented the appearance of 120-kDa FGFR1-EGFP without affecting actin levels. Hence, soluble FGFR1-EGFP may represent a newly synthesized receptor. To verify this we transfected TE671 cells with w.t. FGFR1 or FGFR1-EGFP and pulse-labeled with [³⁵S]methionine for 1 h. After a chase with nonradioactive methionine, cell extracts were isolated and immunoprecipi-

Figure 2. Characterization of FGFR1-EGFP in the S150 soluble cytosolic (S), microsomal membrane P150 (P), and nuclear (N) fractions. (A) TE671 were transfected with FGFR1-EGFP or completely soluble FGFR1 (SP⁺)-EGFP and were analyzed at the indicated times. Immunoblotting performed with anti-FGFR1 McAb6. FGFR1 was not detectable in cells transfected with control pEGFP (unpublished data). FGFR1-EGFP was expressed at 8 h posttransfection. Membrane-associated (150 and 160 kDa), cytosolic and nuclear (120 kDa) FGFR1-EGFP were detected at 12 and 20 h posttransfection (detected with McAb6). (NG) and (G) depict nonglycosylated and different glycosylated forms of receptor, respectively. Subcellular fractions were characterized as described in (Myers *et al.*, 2003) and in the *Materials and Methods*. (B) Full-length FGFR1 and FGFR1-EGFP are present in the membrane (P) and soluble cytosolic (S) fractions of TE671 cells. Cells were transfected with the indicated plasmids. Cell fractions were immunoprecipitated with a C-terminal FGFR1 antibody, control anti-RNA Polymerase II antibody or an EGFP antibody and immunoblotted with a FGFR1 N-terminal McAb6 antibody. (C) Effect of proteasome or protein synthesis inhibition on cytosolic FGFR1-EGFP. TE671 were transfected with FGFR1-EGFP. Twenty-four hours after transfection some dishes were treated for with lactacystine (Lac: 3 μ M, 4 h) or with cycloheximide (Chx: 100 μ g/ml, 4 h). Immunoblotting with FGFR1 McAb6 in S150 fraction is shown. (D) Pulse-chase analysis of FGFR1-EGFP and FGFR1. TE671 cells were transfected with FGFR1, FGFR1-EGFP, or with control pcDNA3.1 vector (v) and labeled 24 h later for 1 h with ³⁵S-cysteine + methionine. The cells were rinsed and then either processed immediately (p, pulse) or incubated for 30, 60, or 120 min as described in *Materials and Methods*. Cell fractions were immunoprecipitated with a GFP (top row) or FGFR1 C-term antibody (bottom row) and analyzed by SDS-PAGE.

tated with FGFR1 or EGFP antibodies (Figure 2D). In cells transfected with the w.t. receptor the first synthesized receptor (detected immediately after labeling) was soluble, 95-kDa, FGFR1 (bottom panel) and in cells transfected with the EGFP tagged receptor the 120-kDa form of FGFR1-EGFP appeared first (top panel). Higher molecular weight forms of FGFR1 and FGFR1-EGFP were detected after an additional 30-min to 2-h chase.

FGFR1 Movement in Different Cytoplasmic Compartments

To determine the mobility of FGFR1, cells expressing FGFR1-EGFP were monitored using a confocal microscope utilizing the FRAP technique. To correctly assess protein mobility by FRAP, photobleaching must be irreversible and fluorescent recovery must reflect the replacement of photobleached molecules with nonbleached molecules (movement of nonbleached proteins into the photobleached area). We confirmed this by demonstrating the absence of recovery in paraformaldehyde-fixed cells expressing FGFR1-EGFP. In fixed cells, bleaching reduced the relative fluorescence, without showing any recovery (Figure 3A), despite that the extent of photobleaching of FGFR1-EGFP was similar in fixed and nonfixed cells. Hence, in our experiments, photobleaching was irreversible and recovery was caused by movement of FGFR1-EGFP into the photobleached area.

As a control for a freely diffusible protein, we used cells expressing nonfused EGFP, which exhibited very fast recovery kinetics, approaching the detection limits of our experimental recording settings (Figure 3B). The EGFP recovery half-time was calculated to be $t_{1/2} = 0.376$ s with a 93% mobile population (Table 1). In contrast, after bleaching live cells that expressed EGFP-tagged FGFR1, the intensity rose more gradually and eventually tapered off to a plateau indicative of steady state movement (Figure 3B).

Previous studies have indicated that FGFR1 exists in two distinct populations (Myers *et al.*, 2003): 1) membrane associated FGFR1, which resides mainly in the cytoplasmic vesicular structures (distinguishable by phase contrast microscopy) in the vicinity of the nucleus, and 2) soluble FGFR1-EGFP, which exists in the peripheral cytoplasm. The first step in our analysis of FGFR1 mobility involved determining if these two compartments had different mobility characteristics. This analysis revealed that in cytoplasmic regions with identifiable ER-Golgi vesicles, only a small fraction ($\approx 24\%$) of the FGFR1-EGFP population showed postbleaching fluorescence recovery (Figure 3C). In contrast, in cytosolic-like regions lacking identifiable ER-Golgi vesicles, $\approx 56\%$ of the FGFR1-EGFP population showed recovery. The differences between compartments were statistically significant ($p < 0.001$). The diffusion rates of both mobile FGFR1-EGFP populations

Figure 3. FRAP analysis of FGFR1-EGFP. A strip across the width of the cell was bleached with high-powered bleach pulse (5–6 mW) and fluorescence was monitored every 0.75 s with a low-powered (20 μ W) scanning beam. (A) Images were collected at the indicated time points before and after bleaching of a 4% paraformaldehyde-fixed cell. The plot confirms the absence of recovery in fixed cells ($n = 3$). Image shows a representative cell. (B) Wild-type FGFR1 distribution and recovery. FGFR1 has a visible specific membrane and soluble distribution. Images were collected at the indicated time points before and after bleaching a cell transfected with w.t. FGFR1-EGFP. Image shows a representative cell. (C) Recovery of specific membranous and soluble populations of w.t. FGFR1-EGFP. In the cytoplasmic regions with identifiable membranous structures, only 25% of the population was mobile, with an average halftime of maximal recovery of 11 s. In the cytoplasm lacking such structures, ~50% of FGFR1-EGFP was mobile with an average $t_{1/2} = 4$ s. Essentially all EGFP molecules showed recovery at $t_{1/2} = 30$ s. Data are the mean of at least 13 cells. Plot, solid lines show the mean value and the dashed line is the 95% confidence interval. Inset, table gives the $t_{1/2}$ and mobile population values obtained from the recovery curves depicted. Errors in table are given as SEM. Differences among FGFR1-EGFP in cytosolic-like compartment, FGFR1-EGFP areas rich in Golgi-ER and nonfused EGFP were statistically significant at $p < 0.0001$.

were significantly ($p < 0.001$) slower than the diffusion rate of nonfused EGFP (Figure 3C).

Cytoplasmic Recovery Influenced by the TMD

Initial FRAP analyses of cytoplasmic FGFR1-EGFP indicated that there are two distinct mobile populations of FGFR1 (in addition to the immobile molecules that show no recovery): 1) a slower-diffusing population associated with membrane-rich regions and 2) a faster diffusing population outside the ER-Golgi membranous structures, which could represent (in

part) the soluble receptor. A difficulty that had to be overcome with transfected FGFR1-EGFP, similar to the endogenous receptor, is that it is distributed in both membranous and soluble populations, in which the limits of each compartment are difficult to dissect using confocal microscopy alone. Therefore, in order to determine whether diffusion rate differences may reflect different ratios of membrane-associated and soluble cytosolic FGFR1-EGFP, we used receptor constructs with mutations that affect the distribution between the membrane and cytosolic compartments. Their mobility was compared with the average mobility of cytoplasmic FGFR1-EGFP (associated and not associated with identifiable ER-Golgi vesicles) that had an intermediate value of 38.5% and a recovery halftime of $t_{1/2} = 8.6$ s (Table 1).

Myers *et al.* (2003), demonstrated that the presence of w.t. FGFR1 in the cytosol (outside of cytoplasmic membranes) critically depends on the receptor's atypical TMD. We have also shown that mutations that increased the number of polar amino acids in the FGFR1 TMD reduced receptor association with membranes, whereas increasing its content in the cytosol and nucleus, in a manner that correlated with increasing TMD hydrophilicity. Therefore, to alter the membrane:soluble protein ratio, we used FGFR1($\Delta 2$) and FGFR1($\Delta 3$) mutants in which two or three hydrophobic to hydrophilic amino acid substitutions were introduced into the TMD (Myers *et al.*, 2003). FGFR1($\Delta 2$), with two hydrophilic amino acid substitutions, displayed a twofold reduction in ER-Golgi association and a similar increase in soluble cytosolic content. FGFR1($\Delta 3$)-EGFP, with three hydrophilic amino acids substitutions introduced into the TMD showed a threefold increase in the cytosol-membrane distribution (Myers *et al.*, 2003). FRAP analysis showed approximately a twofold increase in the size of the FGFR1($\Delta 2$)-EGFP mobile

Table 1. Comparison of diffusion rates and mobile population of FGFR1, FGFR1 mutants, and controls

Protein	Recovery rate $t_{1/2}$ (s)	Mobile population (%)
FGFR1 (n = 21)	8.607 $\pm$ 0.917	38.532 $\pm$ 2.113
FGFR1(SP ⁻) (n = 6)	0.736 $\pm$ 0.141	98.163 $\pm$ 0.724
FGFR1($\Delta 2$) (n = 6)	3.389 $\pm$ 0.827	65.583 $\pm$ 11.068
FGFR1($\Delta 3$) (n = 9)	1.583 $\pm$ 0.281	71.469 $\pm$ 9.879
FGFR1(TM ⁻) (n = 6)	37.712 $\pm$ 3.541	25.431 $\pm$ 1.658
EGFP (n = 13)	0.376 $\pm$ 0.06	92.777 $\pm$ 0.958
p85 α EGFP (n = 9)	2.629 s $\pm$ 0.292	91.55 $\pm$ 2.01

Average values are given for the indicated number of cells, which was calculated by averaging the $t_{1/2}$ or mobility value for each cell fit by nonlinear regression analysis. Error is expressed as SEM $t_{1/2}$ and mobile populations of all FGFR1 mutants, p85 α and nonfused EGFP were significantly different from those of the wild-type FGFR1 ($p < 0.0001$).

Figure 4. Recovery of w.t. FGFR1-EGFP and its diffusion rate in the cytoplasm are affected by membrane insertion. Strip FRAP measurements of FGFR1-EGFP are compared with mutants with TMD alterations [FGFR1(Δ2)-EGFP and FGFR1(Δ3)-EGFP] or lacking the signal peptide (FGFR1(SP⁻)-EGFP). (A) One exponential analysis of w.t. FGFR1 and the indicated mutants. Data are the mean of at least six cells. Plot, solid lines show the mean value and the dashed line is the 95% confidence interval. Specific values are given in Table 1. (B) Three distinct populations of FGFR1 are altered due to domain mutations in FGFR1. The same samples were analyzed as in A but with two exponential analysis of w.t. FGFR1 and the indicated mutants. The fast soluble population is represented by the solid bars and the slow membranous population is represented by the dashed bars. The $t_{1/2}$ values for the fast and slow populations are given in the table below the graph and correspond to the protein directly above the value. ND, not detected. Error in table is given as SEM. Values obtained from strip FRAP measurements (depicted in A) of FGFR1-EGFP, transmembrane mutants (FGFR1(Δ2)-EGFP and FGFR1(Δ3)-EGFP), and SP deletion mutant (FGFR1(SP⁻)-EGFP). The sizes of slow and fast populations of FGFR1 mutants differed significantly compared with w.t. FGFR1 ($p < 0.0001$). Slow diffusion rates of FGFR1 mutants differed significantly from w.t. FGFR1 ($p < 0.001$).

population (65%) and a significant increase in its diffusion rate ($t_{1/2} = 3.4$ s; Figure 4A; Table 1) compared with the average FGFR1-EGFP mobility (38% and $t_{1/2} = 8.6$ s) throughout the cytoplasm. FGFR1(Δ3) displayed a faster diffusion rate ($t_{1/2} = 1.6$ s) and further increased the mobile population (71.5%). Thus, the increase in the relative abundance of soluble FGFR1 increases the highly mobile population and diffusion rate of the receptor within the cytoplasm. To specifically determine the mobility of soluble FGFR1, we transfected TE671 cells with FGFR1(SP⁻)-EGFP, which is a nonmembrane, exclusively cytosolic protein (Myers *et al.*, 2003; also see Figure 2). Deletion of the SP produced a receptor population in which more than 98% of the molecules displayed a high diffusion rate ($t_{1/2} = 0.736$ s).

The shapes of the recovery curves of FGFR1-EGFP, FGFR1(Δ2)-EGFP, and FGFR1(Δ3)-EGFP (Figure 4A) suggested that in each case at least two subpopulations may be present that differed in the diffusion rate. To verify this, the obtained results were subjected to a two-exponential association analysis, that revealed a better fit with two populations rather than one ($R^2 = 0.765$ for one exponential and 0.943 for two exponential association for FGFR1-EGFP). The same phenomenon was observed with FGFR1(Δ2)-EGFP and FGFR1(Δ3)-EGFP, but not with FGFR1(SP⁻)-EGFP which displayed only a one-component recovery curve ($R^2 = 0.918$ for one-exponential and 0.423 for two-exponential association). This analysis distinguished two separate populations with significantly different diffusion rates for FGFR1-EGFP, FGFR1(Δ2)-EGFP, and FGFR1(Δ3)-EGFP (results are summarized in Figure 4B). The faster-moving populations recovered at a rate of $t_{1/2} \leq 1.00$ s, which was not significantly different from the single rate component of FGFR1(SP⁻)-EGFP. The slower recovering populations showed an increased diffusion rate ($t_{1/2}$) with increasing TMD hydrophilicity from ~1 min (FGFR1-EGFP) to 15 s (FGFR1(Δ2)-EGFP) and to <10 s (FGFR1(Δ3)-EGFP; Figure 4B). The size of the fast population was significantly increased with FGFR1(Δ3)-EGFP and FGFR1(SP⁻)-EGFP; however, there was no significant change with FGFR1(Δ2)-EGFP. The size of the slow population was similar for FGFR1 and FGFR1(Δ2)-EGFP, but was significantly reduced with FGFR1(Δ3)-EGFP. The completely soluble FGFR1(SP⁻)-EGFP contained no measurable slow population.

To ascertain that the fast recovery of FGFR1(SP⁻) reflects its diffusion rate in the cytosol rather than the ER lumen, we used the FGFR1(TM⁻) receptor mutant fused to EGFP. Deletion of the TMD caused an initial accumulation of the nonmembrane receptor in the lumen of the ER-Golgi vesicles. When a portion of such an individual vesicle was photobleached, we observed only a partial fluorescence recovery (25%, Figure 5), which was more limited than the average cytoplasmic recovery of FGFR1-EGFP (38%). This indicated that intravesicular FGFR1 may not be readily exchangeable between different vesicles. Additionally, the diffusion rate of FGFR1(TM⁻)-EGFP ($t_{1/2} = 37$ s) was several-fold slower than the average diffusion rate of FGFR1-EGFP and FGFR1(SP⁻)-EGFP. These results indicate that the FGFR1(TM⁻)-EGFP intravesicular soluble pool has a more re-

Figure 5. Intraluminal FGFR1(TM⁻)-EGFP has low mobility. Cells were transfected with FGFR1(TM⁻)-EGFP a nonmembrane protein that accumulates inside the ER vesicles. Arrow points to photobleached cytoplasmic region. Fluorescent intensity is representative of the mobile population. Data are the mean of at least six cells. Specific values are given in Table 1.

stricted mobility than cytosolic FGFR1(SP⁻)-EGFP and confirms that the fast mobile populations of FGFR1, FGFR1(Δ 2), FGFR1(Δ 3), and FGFR1(SP⁻) reside in the cytosol and not in the intravesicular space of the cytoplasm.

To elucidate the possible sources of the two populations of FGFR1, the ER and proteosome were further investigated. Proteosome inhibition via lactacystine treatment showed that the soluble cytosolic FGFR1-EGFP was not a result of proteolytic degradation (Figure 2C). Consistent with these results we found that proteosome inhibition had no significant effect on the mobility of FGFR1-EGFP ($p > 0.05$). Two exponential analysis verified that the highly mobile, fast population of FGFR1-EGFP still existed after 4 h of lactacystine treatment (in addition to the slower, membranous population; Figure 6A), indicating that two dynamic populations of FGFR1-EGFP are not a product of proteosome degradation. An inhibitor of intracellular protein transport to the Golgi complex, BFA, was utilized to determine if the Golgi was the source of the fast, soluble population. One-hour incubation with BFA increased FGFR1-EGFP fluorescence in perinuclear ER-like vesicles and reduced the homogeneous cytosolic and nuclear fluorescence. However, we observed no marked depletion of cell surface-associated FGFR1-EGFP with BFA treatment (Figure 6B). Consistent with these results, treatment with BFA completely abolished the fast FGFR1 population, resulting in only one mobile population with a reduction in the mobile population. The size of the mobile population was reduced to 35%, compared with a mobile population of 65% in cells that did not undergo BFA treatment (Figure 6C). Therefore, inhibition of ER Golgi trafficking prevents the generation of the cytosolic fast FGFR1-EGFP population and increases the size of the immobile population.

RSK1 Influences FGFR1 Mobility

Thus far, our analyses had defined kinetic subpopulations of cytoplasmic FGFR1 and their compartmental associations (membrane vs. cytosolic) by using FGFR1 domain mutants. The next step in this process was to identify mechanism(s) by which FGFR1 mobility could be controlled. FGFR1 is a receptor TK that activates multiple signaling pathways through direct interactions with its TK domain resulting in the subsequent phosphorylation of proteins, such as FRS2 (Ong *et al.*, 2000), PLC γ (Powers *et al.*, 2000), Grb14 (Reilly *et al.*, 2000), etc. Previous work in our laboratory has showed that RSK1 interacts with FGFR1 in both the cytoplasm and nucleus and proposed that RSK1 may play a role in FGFR1 trafficking (Hu *et al.*, 2004). Therefore, the first step in determining a possible mechanism of FGFR1 kinetic regulation was to test whether endogenous FGFR1 and RSK1 are coregulated during cell stimulation. As a model we used BAMC, which express measurable levels of endogenous FGFR1. FGFR1 rapidly accumulates in the nucleus of stimulated BAMC, with little FGFR1 at the cell surface and a small amount in the cytoplasm (Stachowiak *et al.*, 1996a). In BAMC stimulation of AII receptors and depolarization with veratridine (or direct stimulation of cAMP and PKC pathways with forskolin and PMA) synergistically stimulates nuclear FGFR1 accumulation (Stachowiak *et al.*, 1996a; Peng *et al.*, 2001, 2002; Figure 7A). The changes in FGFR1 distribution were accompanied by an increase in cytoplasmic phospho-RSK1 immunoreactivity (IR) and a more profound increase in nuclear phospho-RSK1 IR (Figure 7A). The changes in cytoplasmic and nuclear RSK1 contents were further confirmed by Western immunoblotting with a pan-antibody that recognizes all forms (phosphorylated and nonphosphorylated) of RSK1 (Figure 7B). Nuclear accumulation of FGFR1 was also confirmed by Western immunoblotting in BAMC, which was pre-

Figure 6. ER Golgi fusion, but not protein degradation determines FGFR1 dynamics. (A) Proteosomal degradation is not a factor determining FGFR1 mobility characteristics. FGFR1-EGFP was co-transfected with pcDNA. Equal amounts (1 μ g) of each plasmid were transfected. Strip FRAP measurements of FGFR1-EGFP-pcDNA3.1 compared with FGFR1-EGFP treated with 3 μ M lactacystine for 4 h. Data are the mean at least 12 cells. The sizes of the slow and fast populations of FGFR1-EGFP with or without lactacystine treatment did not differ significantly ($p > 0.05$). (B) ER Golgi fusion required for soluble and nuclear FGFR1. FGFR1-EGFP was cotransfected with pcDNA. Equal amounts (1 μ g) of each plasmid were transfected. Cells were treated for 1 h with brefeldin A (BFA) 24 h posttransfection. Image shows a representative cell. (C) ER Golgi fusion is required for fast, soluble mobility characteristics. FGFR1-EGFP was cotransfected with pcDNA. Equal amounts (1 μ g) of each plasmid were transfected. Strip FRAP measurements of FGFR1-EGFP-pcDNA3.1 compared with FGFR1-EGFP treated with 10 μ g/ml BFA for 1 h. Fluorescent intensity is representative of the mobile population. Data are the mean at least 14 cells. The fast population is completely eliminated, leaving only 1 mobile population. The inset image displays only the mobile population from two exponential analysis of FGFR1-EGFP with and without BFA treatment. The exact same cells were analyzed in both graphs.

Figure 7. RSK1 influences the movement of FGFR1. (A) Coregulation of FGFR1 and RSK1 in BAMC. BAMC were maintained in serum free medium and treated with 0.1 μ M angiotensin II and 20 μ M veratridine for 0 (control) or 4 h. Cells were fixed, permeabilized with 0.5% Triton X-100, and stained with C-term polyclonal FGFR1 antibody or with anti-phospho-RSK1 antibody. Immune complexes were revealed with secondary antibody fused to Alexa 488. Confocal sections approximately through the middle of the nuclei of the representative cells are shown. Similar results were observed in cells treated with 10 μ M forskolin and 0.1 μ M PMA (unpublished data). (B) Nuclear and cytoplasmic extracts from control BAMC or BAMC treated with veratridine and angiotensin (AII) for 4 h were immunoprecipitated with a RSK1 antibody and probed with a RSK1 antibody. (C) Nuclear and cytoplasmic extracts from control or stimulated BAMC were subjected to Western blot analysis with FGFR1 antibody. Some cultures were treated with 10 μ M cycloheximide for the duration of the experiment (+) as previously described (Stachowiak *et al.*, 1994b).

vented by protein synthesis inhibition (Figure 7C). These results support the model in which a newly synthesized receptor translocates to the nucleus, also demonstrated with transfected FGFR1 (Figure 2C). Thus, nuclear FGFR1 is a newly synthesized protein in both primary cells and cell lines.

Given that nuclear FGFR1 accumulation was accompanied by an upregulation of its binding partner (RSK1) and their nuclear coaccumulation, we examined if the increase in cytoplasmic RSK1 content affects FGFR1 mobility. First, we determined if the EGFP tag interferes with the FGFR1-RSK1 interaction by transfecting cells with flag-tagged RSK1 and with FGFR1-EGFP or w.t. FGFR1. The cellular extracts were then immunoprecipitated with anti-Flag or a control antibody. The EGFP tag did not prevent the FGFR1-RSK1 interaction (Figure 8A).

To determine if RSK1 had an influence on FGFR1 mobility, we cotransfected equal amounts of FGFR1-EGFP with RSK1 or with control plasmids. TE671 express endogenous RSK1 and transfection of flag-tagged RSK1 increased the total level of RSK1 approximately 2- to 3-fold (see Figure 8E). The magnitude of this increase was similar to the increase in cytoplasmic RSK1 content in BAMC induced by angiotensin and veratridine. In cells transfected with RSK1-flag, we found an increase in both the mobile population (73%) of FGFR1 and its diffusion rate ($t_{1/2} = 1.27$ s; Figure 8B). Transfection of a N-terminal kinase inactive mutant of RSK1 markedly reduced the effect that RSK1 had on increasing FGFR1 mobility (Table 2). Additionally, in contrast to active RSK1, its kinase inactive mutant had no effect on the diffusion rate of FGFR1. This result demonstrates that RSK1's exo-kinase activity is required for RSK1 to increase FGFR1 mobility and to decrease its diffusion rate. Transfection of CREB-binding protein (CBP; Fang *et al.*, 2005), which interacts with FGFR1 in the nucleus, had no effect on cytoplasmic FGFR1-EGFP mobility (Figure 8B, Table 2).

The specificity of the phenomenon of RSK1 accelerating FGFR1-EGFP movement was further verified via FGFR1(TK⁻), which does not bind to RSK1 (Hu *et al.*, 2004). Deletion of the TK domain [FGFR1(TK⁻)-EGFP] produced a stark acceleration of the diffusion rate ($t_{1/2} = 0.8$ s) without affecting the size of mobile receptor population (Table 2). These results are consistent with biochemical results that show that FGFR1(TK⁻), like w.t. FGFR1, is expressed both as a membrane-associated and soluble receptor (Myers *et al.*, 2003). To confirm that the change in protein size may not account for the fast diffusion rate of FGFR1(TK⁻)-EGFP, we analyzed the mobility of the p85 α sub-

unit of PI3K. Even though the molecular size of FGFR1(TK⁻)-EGFP (90–100 kDa) and p85-EGFP (Ward *et al.*, 1996; Dunham *et al.*, 2004) were similar, p85 α -EGFP, a nonmembrane protein, showed a significantly slower recovery ($t_{1/2} = 2.6$ s) than FGFR1(TK⁻)-EGFP (Tables 1 and 2). Also, more than 90% of the p85 α -EGFP population was mobile, which is consistent with its cytosolic, nonmembrane distribution. These results indicate that the increased mobility of FGFR1(TK⁻) is not a result of its reduced size, but may be a direct result of diminished interactions with other cellular structures and/or complexes. FGFR1(TK⁻)-EGFP's mobility was not influenced by cotransfection of RSK1 (Figure 8C; Table 2). The recovery rate could not be analyzed because of the diffusion rate being below the detection limit of our experimental setup.

The effect of RSK1 on FGFR1 mobility (Figure 8D) was similar to the effects of the FGFR1 mutations that increased its fast, cytosolic population (Figure 4B). Hence, to determine the underlying mechanism of RSK1 action, we examined whether RSK1 may influence FGFR1's distribution between the cytosolic and membranous compartments. An approximately twofold increase in cytosolic RSK1 concentration caused by transfection of RSK1-flag was accompanied by a marked increase in the levels of nonglycosylated FGFR1, both in the cytosol and nucleus. In contrast, the content of glycosylated membrane-associated FGFR1 appeared slightly reduced by RSK1 (Figure 8E). To determine if RSK1's kinase activity is necessary for the translocation of FGFR1 into the cytosol and nuclear compartments the kinase inactive mutant of RSK1 was tested for its effect on FGFR1 distribution. The kinase inactive mutant was unable to cause FGFR1 translocation into the cytoplasm (Figure 8E). Thus, the RSK1-induced increase in the highly mobile FGFR1 population correlated with an increase in the FGFR1 cytosolic and nuclear content.

DISCUSSION

A model in which FGFR1 serves as a transmembrane receptor TK as well as a soluble protein that translocates into the nucleus has previously been proposed. This model has been supported by reports of the presence of FGFR1 in membrane, cytosolic, and nuclear fractions in fixed and live cells (Myers *et al.*, 2003; Stachowiak *et al.*, 1997, 2003b). Based on this model and other studies, a novel signal transduction mechanism was further established in which soluble FGFR1

Figure 8. RSK1 influences the cellular trafficking of FGFR1. (A) Coimmunoprecipitation of RSK1 with FGFR1 in TE671 cells. Equal amounts of FGFR1, FGFR1-EGFP were cotransfected with pcDNA or RSK1-flag. The samples were immunoprecipitated with a Flag

Table 2. Comparison of diffusion rates and mobility of FGFR1-EGFP in the presence of known interacting proteins and controls that do not interact

Protein	Recovery rate $t_{1/2}$ (s)	Mobile population (%)
FGFR1-pcDNA (n = 21)	8.607 $\pm$ 0.917	38.531 $\pm$ 2.113
FGFR1-CBP (n = 9)	9.751 $\pm$ 2.06	49.091 $\pm$ 0.326
FGFR1-RSK1 (n = 30)	1.273 $\pm$ 0.341	73.342 $\pm$ 2.202
FGFR1-RSKmt (n = 21)	9.556 $\pm$ 1.75	54.163 $\pm$ 3.48
FGFR1(TK ⁻)-pcDNA (n = 25)	0.807 $\pm$ 0.119	46.502 $\pm$ 6.585
FGFR1(TK ⁻)-RSK1 (n = 30)	0.764 $\pm$ 0.154	53.037 $\pm$ 6.124

Average values are given for the indicated number of cells, which was calculated by averaging the $t_{1/2}$ or mobility value for each cell fit by nonlinear regression analysis. Error is expressed as SEM. $t_{1/2}$ and mobile population of FGFR1 in the presence of cotransfected RSK1 (but not CBP) were significantly different from FGFR1-pcDNA ($p < 0.001$). No significant differences between FGFR1(TK⁻) cotransfected with control plasmid (pcDNA) or RSK1 were found.

acts as a general nuclear transcription regulator that stimulates multigene programs required for cell growth and differentiation (Stachowiak *et al.*, 1996; Peng *et al.*, 2001, 2002;

antibody and immunoblotted with FGFR1 McAb6 or RSK1 antibody. The lanes correspond to the following transfected plasmids: (1) pcDNA + pcDNA, (2) pcDNA + FGFR1, (3) RSK1-Flag + FGFR1, (4) pcDNA + FGFR1-EGFP, and (5) RSK1-Flag + FGFR1-EGFP. (B) Recovery of w.t. FGFR1-EGFP is facilitated by RSK1, but not kinase inactive RSK1. FGFR1-EGFP was cotransfected with pcDNA, or with plasmids expressing CBP, RSK1, or kinase inactive RSK1 (RSKmt) proteins that interact with nuclear or nuclear and cytoplasmic FGFR1, respectively (Hu *et al.*, 2004). Equal amounts (1 μ g) of each plasmid were transfected. Strip FRAP measurements of FGFR1-EGFP-pcDNA3.1 compared with FGFR1-EGFP cotransfected with known FGFR1-binding proteins. Fluorescent intensity is representative of the mobile population. Data are the mean at least nine cells. Plot, solid lines show the mean value and the dashed line is the 95% Confidence interval. Specific values are given in Table 2. (C) Mobility of FGFR1(TK⁻)-EGFP is not facilitated by RSK1. FGFR1(TK⁻)-EGFP was cotransfected with pcDNA or with RSK1. Equal amounts (1 μ g) of each plasmid were transfected. Strip FRAP measurements of FGFR1(TK⁻)-EGFP-pcDNA3.1 compared with FGFR1(TK⁻)-EGFP cotransfected with RSK1. Fluorescent intensity is representative of the mobile population. Data are the mean at least nine cells. Plot, solid lines show the mean value and the dashed line is the 95% confidence interval. Specific values are given in Table 2. (D) RSK1 increases the fast, soluble population of FGFR1. The fast soluble population is represented by solid bars and the slow membranous population is represented by the dashed bars. The $t_{1/2}$ values for the fast and slow populations are given in the table below the graph and correspond to the protein directly above the value. Error in table is given as SEM. Values obtained from strip FRAP measurements (depicted C) of FGFR1-EGFP and FGFR1-RSK1. Data are the mean of at least 21 cells. The sizes of the slow and fast populations of FGFR1 differed significantly from that in the presence of RSK1 ($p < 0.0001$). The slow diffusion rate of FGFR1 also differed significantly in the presence of RSK1 ($p < 0.0001$), the fast population is below the detection limits of our setup. (E) RSK1 increases the cytosolic and nuclear content of FGFR1 in a kinase-dependent manner. FGFR1 was cotransfected with RSK1, kinase inactive RSK1 (RSKmt), or control pcDNA3.1. The cytosolic, soluble (S150), microsomal (P150), and nuclear fractions were isolated and immunoblotted with FGFR1 McAb6, RSK1, or an actin antibody. The lanes correspond to the following transfected plasmids: (1) pcDNA + FGFR1-EGFP (2) RSK1mt-Flag + FGFR1-EGFP, (3) RSK1-FGFR1-EGFP. NG and G, nonglycosylated and different glycosylated forms of FGFR1-EGFP, respectively.

Fang *et al.*, 2005). To further the knowledge regarding FGFR1 nuclear accumulation, the current investigation was designed to characterize the spatial dynamics regarding FGFR1 cytoplasmic trafficking and therefore the underlying mechanisms that control its nuclear accumulation.

The present study reveals FGFR1 protein processing and shows differences between the membrane, soluble, and nuclear fractions. FGFR1 initially accumulates in the membrane fraction as a processed protein and then later in the nuclear and soluble fractions. During FGFR1 membrane synthesis, the initial protein products have a low molecular weight (95 kDa) due to lack of glycosylation, as shown by Stachowiak *et al.* (1997). The newly synthesized proteins are later processed through the Golgi and undergo glycosylation refinement to give the larger molecular weights observed. Soluble FGFR1 does not contain glycosylated forms, consistent with its cytosolic localization. Nuclear FGFR1 exists in both the glycosylated and nonglycosylated forms. Only nonglycosylated FGFR1 coimmunoprecipitates with CBP (Fang *et al.*, 2005) and thus is likely to represent the intranuclear receptor (Fang *et al.*, 2005). The larger molecular forms of FGFR1 in the nuclear fraction may represent receptor molecules associated with the nuclear membrane, which is continuous with ER membranes.

The dynamics of cytoplasmic FGFR1-EGFP were analyzed in order to gain further insight into mechanisms that determine the distinct FGFR1 populations. Our current investigation shows that in the cytoplasm FGFR1 exists as three major kinetic populations: 1) a relatively "immobile" FGFR1 pool that undergoes no movement within the time frame of the experimental setting (≈ 5 min), 2) slowly diffusing FGFR1-EGFP molecules that recover with a $t_{1/2}$ of ~ 1 min, and 3) a fast diffusing population that restores FGFR1-EGFP fluorescence within 1–2 s. The mobility of FGFR1 mutants with reduced (TMD mutants) or abolished membrane association [FGFR1(SP⁻)] indicate that the immobile and slow moving FGFR1 populations represent transmembrane FGFR1, whereas the fast moving population represents the soluble receptor.

The nature of the immobile FGFR1 population remains to be elucidated. However, an increase in the immobile FGFR1 population caused by BFA treatment suggests that membranous FGFR1 associated with the ER represents an immobile population.

The slow diffusing subpopulation represents the FGFR1 molecules that diffuse within the membrane plane. We show that the rate of FGFR1 diffusion within the membrane is influenced by the TMD interaction with the lipid bilayer; i.e., a faster FGFR1 diffusion was observed with increases in TMD hydrophilicity. This behavior of membrane FGFR1 is similar to that of another related transmembrane protein, EGFR1. EGFR1 mobility analyses in the plasma membrane using a fluorescent ligand showed that it has a relatively slow diffusion rate compared with the similar-sized cytosolic protein (Livneh *et al.*, 1986).

The rapidly diffusing FGFR1 population is the soluble receptor, as indicated by the completely soluble FGFR1 mutant, FGFR1(SP⁻). Proteasome inhibition revealed that this fast population was not a product of degradation, but rather a newly synthesized protein as shown in studies using cycloheximide to block protein synthesis. BFA, which inhibits ER vesicle fusion with the Golgi (Klausner *et al.*, 1992), depleted the fast cytosolic receptor and prevented its nuclear accumulation. Thus, the soluble fast receptor is most likely transported out of the ER into the cytosol and then into the nucleus. However, BFA did not deplete cell surface-associated FGFR-EGFP, indicating that soluble and nuclear

FGFR1 are not derived from the cell surface. This is consistent with the earlier finding that biotinylated cell surface FGFR1 did not accumulate in the nucleus (Stachowiak *et al.*, 1997).

Pulse-chase analysis provides additional information regarding the timing of the generation of fast, soluble FGFR1. The receptor undergoes glycosylation as it passes through the Golgi before reaching the cell surface membrane. Soluble, nonglycosylated FGFR1 appears before glycosylated, membrane-associated FGFR1, which indicates that soluble FGFR1 is generated from a pre-Golgi compartment, possibly the *cis*-Golgi, before the membrane receptor becomes extensively glycosylated (Dean and Pelham, 1990). The exact mechanism of processing of soluble FGFR1 has yet to be determined, but the soluble population may be synthesized without a membrane-inserted intermediate as indicated by the lack of an increase in the soluble form of FGFR1 during the chase.

Analysis of the mechanism(s) that may control FGFR1 kinetics revealed that its interaction with RSK1 significantly influences FGFR1's mobility and diffusion rate. In general, protein interactions with other proteins and cellular structures tend to reduce protein diffusion rates (Lippincott-Schwartz *et al.*, 2001). One such example is a FGFR1-binding protein, p85 α (PI3K subunit), whose mobility was reduced by FGFR1 (Dunham *et al.*, 2004). In contrast, the present study showed that the FGFR1-binding protein, RSK1, markedly increased the highly mobile cytosolic FGFR1 population and its overall diffusion rate and also increased FGFR1 nuclear accumulation. The magnitude of the RSK1-induced changes in cytoplasmic FGFR1-EGFP mobility and diffusion rate were similar to the changes caused by FGFR1 mutations [FGFR1($\Delta 2$ – $\Delta 3$); FGFR1(SP⁻)], which increased the soluble population and decreased the membrane-associated population. Thus, up-regulation of endogenous cytoplasmic RSK1 by stimuli that lead to FGFR1 nuclear accumulation may serve to stimulate the generation of highly mobile, cytosolic FGFR1 that can rapidly enter the nucleus. RSK1's kinase activity could redirect FGFR1 synthesis from the Golgi glycosylation pathway toward its cytosolic extrusion. Such regulation of FGFR1 synthesis by RSK1 is consistent with the known association of RSK1 with ER-attached polyribosomes (Angenstein *et al.*, 1998).

Model

On the basis of our findings, we propose a model in which FGFR1 is synthesized on ER-attached polyribosomes and directed to different subcellular locations. One location would be the Golgi, where some FGFR1 molecules are processed via extensive glycosylation processing to yield membrane bound receptors. In ER vesicles, FGFR1 movement is spatially restricted, but it may gain lateral mobility as the receptor enters the Golgi and/or larger continuous membrane systems, such as the plasma membrane. In addition to this "membrane pathway," newly-synthesized nonglycosylated FGFR1 may exit pre-Golgi vesicles into the cytosol in a process facilitated by RSK1's kinase activity. Generation of this fast cytosolic receptor yields highly mobile FGFR1 molecules that can readily diffuse within the cytoplasm. Such diffusion is likely to be essential for an FGFR1 interaction with the nuclear pore complex and nuclear translocation of associated proteins as described by Reilly and Maher (2001). RSK1's regulation of the mobile FGFR1 population and diffusion rate may provide a physiological mechanism for nuclear FGFR1 accumulation. In addition, RSK1 contains a NLS and could serve as a nuclear chaperon for FGFR1. This process, along with the stimulation of RSK1 kinase activity

by FGFR1, could account for nuclear FGFR1's modulation of RSK1's activation of CREB-mediated gene expression (Hu *et al.*, 2004). Thus, RSK1 and FGFR1 may act in concert to modulate transcriptional events accompanying cell development.

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