Fibroblast Growth Factor Receptor-1 (FGFR1) Nuclear Dynamics Reveal a Novel Mechanism in Transcription Control

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Nuclear FGFR1 acts as a developmental gene regulator in cooperation with FGF-2, RSK1, and CREB-binding protein (CBP). FRAP analysis revealed three nuclear FGFR1 populations: i) a fast mobile, ii) a slower mobile population reflecting chromatin-bound FGFR1, and iii) an immobile FGFR1 population associated with the nuclear matrix. Factors (cAMP, CBP) that induce FGFR1-mediated gene activation shifted FGFR1 from the nuclear matrix (immobile) to chromatin (slow) and reduced the movement rate of the chromatin-bound population. Transcription inhibitors accelerated FGFR1 movement; the content of the chromatin-bound slow FGFR1 decreased, whereas the fast population increased. The transcriptional activation appears to involve conversion of the immobile matrix-bound and the fast nuclear FGFR1 into a slow chromatin-binding population through FGFR1's interaction with CBP, RSK1, and the high-molecular-weight form of FGF-2. Our findings support a general mechanism in which gene activation is governed by protein movement and collisions with other proteins and nuclear structures.

INTRODUCTION

A novel gene regulatory mechanism, integrative nuclear FGF receptor-1 signaling (INFS), has been shown to control cell development (Stachowiak *et al.*, 2007). In INFS, activation of cell surface neurotransmitter, hormonal or growth factor receptors, and their intracellular messengers (i.e., cAMP) stimulates the release of FGF receptor-1 (FGFR1) from pre-Golgi membranes into the cytosol. The receptor and its ligand, FGF-2, are cotransported into the nucleus by a mechanism that involves importin β and engage in the regulation of genes at different chromosomal loci (Reilly and Maher, 2001; Stachowiak *et al.*, 2003b). The INFS signaling mechanism involves FGFR1 feeding forward these signals to CREB-binding protein (CBP), a common and essential transcriptional coactivator that acts as a gene activation gating factor (Myers *et al.*, 2003; Fang *et al.*, 2005; Stachowiak *et al.*,

2007). Nuclear FGFR1 executes the release of CBP from its inactive complex with RSK (Fang *et al.*, 2005), a process shown to up-regulate gene activities associated with cell differentiation (Nakajima *et al.*, 1996). Through the coupled activation of CBP by INFS and transcription factors by specific signaling pathways, this signaling mechanism may enable coordinated gene activation by developmental cues and has been referred to as a “feed-forward-and-gate” signaling (Fang *et al.*, 2005; Stachowiak *et al.*, 2007). Coextraction of FGFR1 with the nucleoplasm, chromatin-associated factors, and the nuclear matrix (NM) as well as its association with active RNA transcription sites and gene promoters indicated both a global and direct role for FGFR1 in gene regulation. Steady-state biochemical analyses suggested that stimulation of gene activities by nuclear FGFR1 occurs in cooperation with CBP as well as nuclear high-molecular-weight (HMW; 23 kDa) FGF-2 and RSK1 and may involve FGFR1 interaction with these proteins (Fang *et al.*, 2005; Stachowiak *et al.*, 2007).

In recent years a dynamic picture of transcription regulation has been emerging based on the findings generated from fluorescence recovery after photobleaching (FRAP; Phair and Misteli, 2000; Phair *et al.*, 2004). The assembly of transcriptional subunits into effective complexes appears to be a dynamic process involving the random collision of the factors involved in transcription (McNally *et al.*, 2000; Karpova *et al.*, 2004; Stavreva *et al.*, 2004). In our previous report we used FRAP to analyze FGFR1 movement and cytoplasmic processing in live cells. There are three distinct pools of the receptor: an immobile pool associated with ER-Golgi vesicles, a slow-moving population ($t_{1/2} = 69$ s) associated with cellular membranes, and a fast-moving ($t_{1/2} = 0.2$ s) cytosolic pool not associated with membranes (Dunham-

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Abbreviations used: CBP, CREB-binding protein; dBcAMP, dibutyryl cAMP; EGFP, enhanced green fluorescent protein; FGF-2, fibroblast growth factor-2; FGFR1, fibroblast growth factor receptor-1; FRAP, fluorescent recovery after photobleaching; FRET, Förster resonance energy transfer; RSK1, p90 ribosomal S6 kinase-1; TK, tyrosine kinase.

Ems *et al.*, 2006). The latter of these likely represents the pool of FGFR1 able to enter cell nucleus (Myers *et al.*, 2003).

The present study used a combination of biophotonics and cell biology tools to further elucidate the mechanisms of gene regulation by the INFS. These tools have provided novel insights into the dynamic nature and molecular proximity of developmental gene regulation. Transcriptional activation by nuclear FGFR1 involves conversion of the immobile NM-bound population and the rapidly diffusing nucleoplasmic population of FGFR1 into a slow chromatin-binding population. FGFR1 transcriptional function is regulated via dynamic associations with the nuclear architecture that is controlled by interactions with CBP, FGF-2, and RSK1.

MATERIALS AND METHODS

Plasmids

pcDNA 3.1, pcDNA3.1-FGFR1, pEGFP-N2, pFGFR1-EGFP, pCMV-RSK1-flag, pRc/RSV-mCBP, and pBI-G expressing anti-sense CBP RNA were described in Fang *et al.* (2005). Plasmids expressing HMW (23 kDa) and low-molecular-weight (LMW; 18 kDa) FGF-2 were described in Claus *et al.* (2003). HMW-FGF-2-EYFP, HMW-FGF-2-ECFP, LMW-FGF-2-EYFP, and LMW-FGF-2-ECFP were generated by subcloning with NheI/EcoRI (HMW) and EcoRI/HindIII (LMW), respectively, from the previously described vectors pEGFP-23 and pEGFP-18 (Claus *et al.*, 2003) into pEYFP and pECFP. FGFR1-ECFP (-EYFP) were constructed by enhancing green fluorescent protein (EGFP) from the FGFR1-EGFP construct and cloning the enhanced cyan fluorescent protein (ECFP) and enhanced yellow fluorescent protein (EYFP), respectively.

Antibodies

FGFR1 (C-terminal), actin, and tubulin, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The N-terminal FGFR1 mcAb6 was described in (Hanneken *et al.*, 1995). FGFR1 rabbit polyclonal antibody Flg(c-15) was purchased from Santa Cruz Biotechnology and Flag monoclonal Ab from Sigma-Aldrich (St. Louis, MO). The polyclonal rabbit GFP antibodies used for immunostaining were from Chemicon (Temecula, CA) and from Clontech Laboratories (Palo Alto, CA). Rabbit anti-Matrin-3 antibody for Western blots was from Bethyl Laboratories (Montgomery, TX), and chicken anti-Matrin-3 antibody (Nakayasu and Berezney, 1991) used for immunocytochemistry was a gift from D. R. Berezney (State University of New York, Buffalo, NY). Specificity of immunostaining was ascertained with control reactions in which the primary Ab was omitted or replaced with preimmune sera or by neutralizing the antibody with cognate peptide (Stachowiak *et al.*, 2003b).

Cell Culture and Transfection

Human TE671 cells and neuroblastoma BE(2)C (Lee and Kim, 2004) were cultured and transfected as previously (Hu *et al.*, 2004; Fang *et al.*, 2005; Dunham-Ems *et al.*, 2006). The efficiency of TE671 transfection was greater than 70% as in our previous studies. For drug treatment the medium was supplemented with 3 μ M lactacystin (Calbiochem, La Jolla, CA) for 4 h (Myers *et al.*, 2003), 100 μ M dBcAMP (Sigma-Aldrich) for 4 h (Stachowiak *et al.*, 2003a), or 10 μ M cytochalasin D (Cyt D; Sigma-Aldrich) for 4 h (Favier *et al.*, 2001) before analysis. Treatments with Act D (actinomycin D; 1 μ g/ml; Sigma-Aldrich) and with DRB (5,6-dichlorobenzimidazole 1- β -D-ribofuranoside; 100 μ M; Sigma-Aldrich) were carried out for 60 min.

Sequential *in situ* cell fractionation and the characteristics of subnuclear fractions were described in He *et al.* (1990) and Stefan Wagner (2003) and in our earlier studies (He *et al.*, 1990; Stachowiak *et al.*, 1996a; Somanathan *et al.*, 2003; Stefan Wagner, 2003). Remaining nuclei after nucleoplasmic and loosely attached proteins were washed away by rinsing with ice-cold 0.5% Triton X-100 containing buffer (10 mM Pipes, 300 mM sucrose, 100 mM NaCl, 3 mM MgCl₂, 10 μ M leupeptin, 10 μ M pepstatin A, 50 μ M bestatin, 1 mM PMSF, and 20 U/ml RNasin) and are referred to as NTX. To *in situ* isolate the NM, chromatin was extracted with the extraction buffer (250 mM ammonium sulfate, 300 mM sucrose, 10 mM Pipes, pH 6.8, 3 mM MgCl₂, 0.5% Triton X-100, and protease inhibitors) and removed by digestion (60 min, room temperature) with RNase-free DNase I (Sigma-Aldrich; 400 U/ml) in digestion buffer (50 mM NaCl, 300 mM sucrose, 10 mM Pipes, pH 6.8, 3 mM MgCl₂, 0.5% Triton X-100, protease inhibitors, and 20 U/ml RNasin). The resulting NM was washed with the extraction buffer. Isolation of the NM was verified by the removal of DNA (DAPI staining) and retention of matrin-3 protein, known to be concentrated in the NM (Hakes and Berezney, 1991).

Cell Lysis Immunoprecipitation and Western Immunoblotting

Isolation and characterization of nuclear and cytoplasmic fractions, protein immunoprecipitation, and immunoblotting were done as described in (Fang *et al.*, 2005; Dunham-Ems *et al.*, 2006).

Sequential fractionation and the characterization of subnuclear fractions was described in (He *et al.*, 1990; Wagner, 2003) and in our earlier studies (He *et al.*, 1990; Stachowiak *et al.*, 1996a; Somanathan *et al.*, 2003; Stefan Wagner, 2003). Nuclei were washed with 1% Triton X-100 buffer containing 50 mM HEPES, 50 mM NaCl, 5 mM EDTA, and protease inhibitors to remove nucleoplasmic proteins. Subsequently protein bound to chromatin were extracted for 60 min with CSK buffer at 4°C. The remaining pellet was washed with 250 mM ammonium sulfate, 300 mM sucrose, 10 mM PIPES, pH 6.8, 3 mM MgCl₂, and 0.5% Triton X-100 containing protease inhibitors. The NM was isolated by digestion with RNase-free DNase I (400 U/ml; 60 min at room temperature) followed by extraction in extraction buffer as described for *in situ* cell fractionation. The CSK-extracted and NM-associated proteins were analyzed by immunoblotting with FGFR1 and matrin-3 antibodies.

Immunocytochemistry

Intact cells or *in situ* isolated cell fractions were fixed with 4% paraformaldehyde for 30 min. Cells were permeabilized with 0.5% Triton X-100. At each stage of *in situ* extraction the cells were stained with DAPI (Sigma-Aldrich) and immunostained with anti-FGFR1 mc Ab6 or rabbit anti-EGFP Ab (to detect FGFR1-EGFP) and with chicken anti-Matrin-3 Ab. Secondary Cy3- or Alexa 488-conjugated antibodies were used (Invitrogen, Carlsbad, CA; Fang *et al.*, 2005). Staining was observed using Zeiss Axioimager Z1 (Thornwood, NY) or Leica TCS-SP2 AOBs (Deerfield, IL). The intensity of FGFR1 mcAb6-immunofluorescent pixels inside the nucleus was determined using ImageJ imaging software (NIH; <http://rsb.info.nih.gov/ij/>). For that purpose the original 16-bit images were used. The border of the nuclei was identified in DAPI-stained cells and in phase-contrast images (examples shown in Supplemental Figures S1 and S2). The threshold for FGFR1 immunofluorescence was set using TE671 cultures transfected with the β -galactosidase plasmid. These cells express endogenous FGFR1 below mcAb6 detection limits and display only background levels of autofluorescence. ImageJ verified that the intensity of FGFR1 immunofluorescence was below saturation levels in all cells measured. ANOVA followed by least-squares difference (LSD) test were applied to analyze differences among cell treatments.

FRAP Image Acquisition and Data Analysis

FRAP analysis was performed according to Bastiaens *et al.* (1996) and Dunham *et al.* (2004, 2006), using the Leica SP2 and Zeiss 510 Meta confocal laser scanning microscopes and the 488-nm Ar laser line as described previously (Dunham-Ems *et al.*, 2006). Levels FGFR1-EGFP fluorescence in individual transfected cells were similar, and cells were randomly selected for the FRAP measurements. FRAP images were acquired every 0.5 s for 1.5 min, then every 1.5 s for an additional 2 min, and finally every 3 s for 5 min using a 63 $\times$ 1.4 NA oil immersion objective. Images were collected using the standard software for the respective microscopes.

The acquired images were analyzed using ImageJ imaging software. 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. The mobile fraction was determined by comparing the relative fluorescence intensity in the bleached region after full recovery (F_{∞}) with the relative fluorescence intensity just before (Phair *et al.*) and after (F_0) bleaching. The mobile fraction (R) is defined by the following equation

$$R = ((F_{\infty} - F_0)/(F_i - F_0))$$

Recovery measurements were quantified by fitting normalized fluorescence intensities of the bleached areas to a one-phase exponential association by using the one exponential nonlinear regression algorithm of Prism 4 (Graph-Pad 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 Origin 6.1 (OriginLab, Northampton, MA). ANOVA and Bartlett tests were applied to analyze differences among recovery half-times ($t_{1/2}$), populations of mobile FGFR1-EGFP and its mutants, and the effects of cotransfected proteins.

Fluorescence Resonance Energy Transfer Experiments

TE671 cells were transfected with pairs of plasmids: (a) FGFR1-YFP + 18 FGF-2-CFP; (b) FGFR1-CFP + 23 FGF-2-YFP; (c) 18 FGF-2-CFP + 23 FGF-2-YFP; negative control pairs (d) FGFR1-CFP + YFP; (e) FGFR1-YFP + CFP; 18 FGF-2-CFP + YFP; 23 FGF-2-YFP + CFP; and positive control (f) fused CFP-YFP in which the CFP and YFP proteins were separated by two amino acids. The mobility of transfected FGFR1 and FGF-2 proteins in live cells influenced the Förster resonance energy transfer (FRET) estimates. Therefore, before FRET imaging, transfected cells were fixed briefly (5 min) with a low concentration (2.5%) of paraformaldehyde (5 min) followed by imaging of

CFP and YFP fluorescence. Confocal FRET imaging was done at 442- and 514-nm excitations for CFP (donor) and YFP (acceptor), respectively. The irreversible acceptor bleaching (Bastiaens *et al.*, 1996; Kenworthy and Edidin, 1998; Periasamy, 2001) was performed with 100% laser power of 514-nm laser line. Forty hours after transfection cells were fixed. FRET imaging was performed using a Leica TCS-SP2-AOBS confocal microscope. The spectrally tunable emission filters were set at 460–500 nm for CFP channel and 520–560 nm for YFP channel. The acceptor bleaching technique (Bastiaens *et al.*, 1996; Kenworthy and Edidin, 1998; Periasamy, 2001) was performed by irreversibly bleaching the acceptor molecules of selected region of interest area (ROI) and the increase in donor emission signal was recorded. In our studies, 100% of the power of the 514-nm laser line was utilized to irreversibly bleach the YFP molecules in the selected area. An increase in the donor signal (within the linear range of measurement) in the bleached area indicates a positive FRET and FRET efficiency can be estimated as $E = 1 - (F_{DA}/F_D)$, where F_{DA} is the donor signal before acceptor bleaching (or donor signal in presence of acceptor) and F_D is the fluorescence signal after acceptor bleaching (or donor signal in absence of acceptor). The confocal imaging software from Leica provided all the required controls for selecting ROIs as well as fast switching of the excitation laser lines or laser intensity for bleaching the selected areas. The

same software was used for image processing as well as to calculate FRET efficiencies using the above equation. Cells transfected with noninteracting 23 FGF-2-YFP + 18 FGF-2-CFP or only CFP or YFP fusion proteins were used as controls. Similar imaging and bleaching conditions were used for the control samples, and no increased signal in the donor channel was observed.

RESULTS

Association of FGFR1-EGFP with Subnuclear Compartments

Transfected chimeric FGFR1-EGFP and its mutants have shown the same subcellular distributions and functions in cell differentiation and gene regulation as the nonfused receptors and have been shown to mimic specific aspects of endogenous FGFR1 biology (Stachowiak *et al.*, 2003b). TE671 medulloblastoma cells were used in the present study be-

Figure 1. Transfected FGFR1-EGFP is expressed at similar levels and is regulated as the endogenous FGFR1. (A) Levels of transfected FGFR1-EGFP in TE671 cells and endogenous FGFR1 in mouse brain. In a typical TE671 transfection experiment 70% or more cells expressed EGFP (not shown). Western blot: lane 1, nontransfected TE671 cells; lane 2, TE671 cells transfected with FGFR1-EGFP; lane 3, mouse brain (postnatal day 4) extract. Each lane contains 30 μ g proteins loaded on the same gel. Blots were probed with anti-FGFR1 (R1) mcAb6 or anti- β -actin Ab. Bands represent different glycosylation forms of FGFR1 and FGFR1-EGFP (Myers *et al.*, 2003). The shift of FGFR1-EGFP bands is attributed to 27-kDa EGFP. β -Actin is used as a reference for the amount of loaded cellular proteins. (B) Endogenous FGFR1 neuroblastoma cells were incubated in control medium or with 0.1 mM dBcAMP (cAMP, 4 h). Cells were fixed with 4% paraformaldehyde for 20 min. Endogenous FGFR1 (neuroblastoma) cells were detected with N-terminal FGFR1 mcAb6 + Alexa 488-conjugated rabbit-antimouse secondary antibody (green). Examples of DAPI (blue) nuclei staining of the same cells are shown. (C) Transfected FGFR1-EGFP (R1-EGFP) - TE671 cells were transfected with FGFR1-EGFP and with empty pcDNA3.1 vector or with 18-kDa FGF-2 or 23-kDa FGF-2-expressing plasmids. Control TE671 cultures transfected with β -galactosidase plasmid alone (B-gal) show low background fluorescence. Twenty four hours after transfection, some of FGFR1-EGFP + pcDNA3.1-transfected cultures were treated with 0.1 mM dBcAMP for 4 h. Cells were fixed and stained along with the neuroblastoma cells (B). Fixation with 4% paraformaldehyde for 30 min diminished native FGFR1-EGFP fluorescence to background levels (not shown). Green fluorescence in TE671 cells reflects mcAb6 + secondary antibody-Alexa 488 complex. The TE671 and neuroblastoma cells were processed in parallel and imaged using the same microscope settings. Examples of DAPI-stained TE671 are presented in Figure 2, A–C. Scale bar, 25 μ m. (D) The FGFR1-IR pixel intensity was measured in the nuclei of randomly selected cells in three separate dishes treated as described in B and C. Threshold was set at the fluorescence levels in TE671 cultures transfected with the β -galactosidase plasmid. Bars represent an average intensity of FGFR1 mcAb6-immunoreactive (IR) pixels ($\pm$ SEM) in 16 nuclei. dBcAMP significantly increased the intensity of nuclear FGFR1-mcAb6-IR pixels in neuroblastoma (endogenous FGFR1) and in TE671 cells (transfected FGFR1-EGFP). ANOVA: $^{++}p < 0.00001$. LSD: different from B-Gal+R1 $^{*}p < 0.05$, $^{**}p < 0.0001$; different from neuroblastoma control $p < 0.0001$. No significant difference between FGFR1-mcAb6-IR in control neuroblastoma and in B-Gal+R1 TE671 cells was observed. Similar results were obtained in three independent experiments.

cause endogenous FGFR1 is below immunocytochemical detection limits, thus allowing us to monitor transfected FGFR1-EGFP with an FGFR1 mcAb6 antibody (Hanneken *et al.*, 1995) in fixed cells. To use FGFR1-EGFP as a model to analyze movement of nuclear FGFR1 in live cells, it was essential to determine whether transfected levels of FGFR1-EGFP were similar to the endogenous levels of FGFR1 typically expressed in neuronal cells. FGFR1-EGFP levels detected in TE671 cells were similar to endogenous FGFR1 in mouse brain extracts using FGFR1 mcAb6 (Figure 1A). As an additional reference, we used the human neuroblastoma cell line BE(2)C (Lee and Kim, 2004), which expressed endoge-

nous FGFR1 at levels similar to cultured bovine adrenal medullary cells, human, astrocytes, neural stem/progenitor cells, or the mouse brain stem cells *in vivo* (Stachowiak *et al.*, 2003a,b; Fang *et al.*, 2005). The levels of transfected FGFR1-EGFP in TE671 cells were similar to endogenous FGFR1 in neuroblastoma cells (Figure 1, B–D). Furthermore, nuclear FGFR1-EGFP accumulation was increased by dibutyryl cAMP (dBcAMP) treatment similar to endogenous FGFR1 (Figure 1, B–D). Cotransfection of the FGFR1 ligand HMW (23 kDa) FGF-2, but not LMW (18 kDa) FGF-2, increased the nuclear content of FGFR1-EGFP (Figure 1, B–D), also similar to endogenous FGFR1 (Stachowiak *et al.*, 2003b).

Figure 2. FGFR1 association with subnuclear compartments and interaction with actin. (A–C) In situ sequential fractionation of TE671 cells. Subnuclear distribution of FGFR1-EGFP is influenced by interaction with actin and by proteasome activity. FGFR1-EGFP-expressing cells were treated with drug-free medium (control) or with cytochalasin D (Cyt D) or lactacystin (Lac). NTX represents nuclei from which nucleoplasm was washed away with Triton X-100 containing buffer (see *Materials and Methods*). NM represents the isolated (DNA free) nuclear matrix. At each stage of extraction the cells were stained with DAPI (blue DNA staining), anti-EGFP Ab + Cy3-conjugated secondary Ab to detect transfected FGFR1-EGFP (red) and anti-Matrin Ab + Alexa 488 (green). Fixation with 4% paraformaldehyde for 30 min diminished native

FGFR1-EGFP fluorescence to low background levels (not shown). Green fluorescence in TE671 cells reflect Matrin-3 Ab + Alexa 488. Association of FGFR1 with the nuclear matrix was also shown by subfractionation of the isolated nuclei (Maher, 1996; Stachowiak *et al.*, 1996a,b; see also Figure 4D). Scale bar, 25 μ m. Larger magnification is shown on Supplemental Figure S2. (D) FGFR1 interacts with actin, but not with tubulin, in the nucleus. TE671 cells were transfected with pcDNA3.1 or FGFR1 (R1) expressing vector. Nuclear fraction was immunoprecipitated (IP) with an actin (Act) or tubulin (Tub) antibody and immunoblotted (IB) with Act (lanes 1 and 2), Tub (lane 3), or FGFR1 (R1) mcAb6 (lanes 4–6). Specificity of FGFR1 coimmunoprecipitation by Act (lane 4) is illustrated by the lack of FGFR1 coimmunoprecipitation by Tub (lane 6). Also, FGFR1 bands were not detected in Act immunoprecipitates from pcDNA-transfected cells that did not express FGFR1 (lane 5). In a separate gel (lanes 7–10) we compared the amounts of transfected FGFR1 (detected with mcAb6) that were immunoprecipitated with an excess of FGFR1 C-term antibody (R1ct, lane 8) or coimmunoprecipitated by Act (lane 10). Those amounts were similar indicating that large amounts of nuclear FGFR1 are associated with actin.

Next we examined whether FGFR1-EGFP associated with the same subnuclear compartments (chromatin and NM) in which endogenous FGFR1 was previously found (Stachowiak *et al.*, 2003b; Fang *et al.*, 2005). We used in situ nuclear, fractionation which allowed concurrent assessment of the subnuclear fractions and FGFR1 in the same cells (Figure 2A; for higher magnification see Supplemental Figure S2). Transfected FGFR1-EGFP was detected with EGFP Ab in the nuclei after washing away soluble and loosely bound nuclear proteins (NTX: nuclei washed with Triton X-100 containing buffer). FGFR1-EGFP immunoreactivity (IR) was still detectable in the NM after chromatin digestion with DNase I, albeit diminished, indicating that the receptor is associated with the NM as well as chromatin. These results were further elaborated by Western blot analysis (see Figure 4D) showing that significantly greater amounts of transfected FGFR1 were associated with the NM than chromatin. These results were also consistent with the previous findings (chromatin immunoprecipitation, in situ fractionation, and isolation of biochemical subnuclear fractions) that the endogenous FGFR1 coassociates with the NM as well as with DNA-bound proteins (Maher, 1996; Stachowiak *et al.*, 1996a,b; Fang *et al.*, 2005).

FGFR1-EGFP NM binding was confirmed by depolymerizing actin filaments, a component of the NM (Nguyen *et al.*, 1998). Cell incubation with Cyt D reduced matrin-IR and abolished FGFR1-EGFP IR in the NM (fraction II; Figure 2B). Thus, actin depolymerization affected the structure of the NM and prevented FGFR1-EGFP's association with the matrix. FGFR1-EGFP was still detected in washed nuclei (NTX), indicating that actin depolymerization did not prevent FGFR1-EGFP's association with chromatin. Coimmunoprecipitation experiments verified that FGFR1 interacts with actin but not with tubulin in the nuclear fraction (Figure 2D).

The results indicated that relatively large amounts of nuclear FGFR1 interact with actin and are consistent with the presence of FGFR1 in the NM.

Nuclear proteasomal activity is associated with the NM (Stenoien *et al.*, 2001b) and degrades proteins engaged in transcription and histone modification (Ezhkova and Tansey, 2004; Osley, 2004). We found that cell treatment with the proteasome inhibitor lactacystin reduced FGFR1-IR associated with the NM (fraction II, Figure 2C). In contrast, FGFR1-EGFP in fraction Nuc(np-) I was clearly detected. Thus, proteasomal activity may promote FGFR1 binding to the NM and perhaps also a chromatin interaction.

Immobile Population Represents Nuclear Matrix-bound FGFR1

To determine whether nuclear FGFR1 displayed recovery kinetics different from those observed with cytoplasmic FGFR1 (Dunham-Ems *et al.*, 2006), cells expressing FGFR1-EGFP were monitored using confocal microscopy by means of FRAP. The recovery of the freely diffusible EGFP protein itself was monitored as a reference. EGFP exhibited very fast recovery kinetics in the nucleus (Figure 3A), as well as in the cytoplasm (Dunham-Ems *et al.*, 2006). Analyses revealed the nuclear EGFP recovery half-time ($t_{1/2}$) was <0.2 s, with a 97% mobile (recovering) population. Other investigators have reported the EGFP recovery time to be ~ 0.05 s (Sprague *et al.*, 2004), which is beyond the resolution of our current experimental setup. Still, in our experiments, nuclear FGFR1-EGFP displayed at least a 10-fold slower recovery rate ($t_{1/2} = 2.2$ s) than EGFP. The nuclear FGFR1 $t_{1/2}$ was significantly shorter than of the cytoplasmic FGFR1 $t_{1/2}$ ($t_{1/2} = 8.61$ s; Dunham-Ems *et al.*, 2006). Approximately 40% of nuclear FGFR1-EGFP remained immobile (i.e., did not recover during the 5-min experiment).

Figure 3. FRAP of FGFR1-EGFP and EGFP in the nucleus. (A) Strip FRAP measurements of TE671 cells transiently transfected with FGFR1-EGFP (R1-EGFP) or nonfused EGFP. Photographs show an example of FGFR1-EGFP-expressing cells before and after photobleaching (N, nucleus; C, cytoplasm). Plot: the shapes represent original data, the solid line represents curve fit with one-exponential analysis, and the dashed line is the 95% confidence interval of the curve fit ($n = 49$). Scale bar, 10 μ m. (B) FRAP of FGFR1-EGFP displays two-exponential recovery kinetics. Example of strip FRAP measurements of nuclear fluorescence in TE671 cells transiently transfected with FGFR1-EGFP. Plot: squares represent original data, red solid line represents curve fit with one-exponential analysis ($R^2 = 0.651$), and blue solid line represents curve fit with two-exponential analysis ($R^2 = 0.989$). (C) Two-exponential analysis of nuclear and cytoplasmic FGFR1-EGFP FRAP recovery in the same transfected TE671 cells. Top, bars represent % of the fast, slow, and immobile FGFR1-EGFP population. Bottom, the $t_{1/2}$ values for the fast and slow populations ($n \geq 40$). The relative sizes of slow and fast populations and recovery half-times of nuclear FGFR1-EGFP differed significantly from the cytoplasmic FGFR1-EGFP EGFP ($p < 0.0001$). (D) Actin and proteasome activity restrict nuclear FGFR1-EGFP FRAP mobility. Cells were

treated with drug-free medium (control) or with cytochalasin D (Cyt D) or lactacystin (Lac). Both treatments significantly reduced the immobile population and increased the overall mobile FGFR1-EGFP population ($p < 0.001$). Cyt D and Lac increased significantly the slow $t_{1/2}$ recovery and Lac also the fast $t_{1/2}$ recovery; $p < 0.001$.

Like our previous results with cytoplasmic FGFR1-EGFP, we obtained a better fit using a bimodal analyses of the FGFR1-EGFP recovery curves ($R^2 = 0.989$), indicating the presence of two mobile nuclear FGFR1-EGFP populations (Figure 3B) rather than one ($R^2 = 0.651$). Similar to cytoplasmic FGFR1, the nuclear receptor contains a hyperdynamic (fast), hypodynamic (slow), and immobile populations (Figure 3C). However, the fast (46%, $p < 0.0001$) and immobile FGFR1-EGFP (36%, $p < 0.0001$) populations in the nucleus are significantly larger, and the slow population (17%, $p <$

0.0001) is significantly smaller than that observed in the cytoplasm (Figure 3C). Furthermore, the recovery half-time of the slow population in the nucleus ($t_{1/2} = 24.41 \pm 0.22$ s) is significantly faster than in the cytoplasm ($t_{1/2} = 69.00 \pm 7.38$ s, $p < 0.0006$).

To verify the presence of fast population we also evaluated the loss of fluorescence that occurred outside of the bleach zone in a number of cells during the bleach. The decrease amounted to $37 \pm 9\%$ of the prebleach fluorescence intensity and occurred within 0.5 s ($n = 6$). These numbers

Figure 4. Transcription activation and inhibition affect FGFR1 association with subnuclear compartments and mobility. (A) FRAP of nuclear FGFR1-EGFP (R1-EGFP) in control and dBcAMP-treated cells (0.1 mM, 4 h; cAMP). dBcAMP treatment eliminated the immobile population and significantly increased the slow population ($p < 0.001$). The slow recovery half-time ($t_{1/2}$) was significantly increased with cAMP treatment ($p < 0.001$, $n \geq 14$). (B) Effects of dBcAMP and transcription inhibitors on nuclear FGFR1-EGFP (R1-EGFP) mobility. FGFR1-EGFP transfected cells were incubated in control medium or with dBcAMP, and with actinomycin D (Act D) or DRB (FRAP ≥ 5 –8 cells/condition). (C) FGFR1-EGFP was cotransfected with plasmids expressing full-length CREB-binding protein (CBP) and its antisense RNA (asCBP; Fang *et al.*, 2005). Total amount of cotransfected DNA was kept constant with pcDNA3.1. CBP significantly increased FGFR1's slow population and recovery half-time, while decreasing the immobile and fast populations ($p < 0.001$). Cotransfection of the antisense CBP significantly reduced the slow population of FGFR1-EGFP ($p < 0.001$, $n \geq 24$ cells). Depletion (80%) of endogenous CBP by asCBP was shown in Fang *et al.* (2005). (D) Effect of dBcAMP, Cyt D, and CBP on FGFR1 association with subnuclear fractions. TE671 were transfected with pcDNA3.1-FGFR1 and were treated with dBcAMP (0.1 mM, 4 h; cAMP) or Cyt D (10 μ M, 4 h; Cyt D; left gel). In a separate experiment (right gel) TE671 were transfected with CBP-expressing plasmid or with control vector. Isolated nuclei were washed in 1% Triton buffer to remove soluble proteins. Chromatin-associated proteins were extracted with CSK buffer and electrophoresed on the same gel along with nuclear matrix (NM)-associated protein and were immunoblotted with FGFR1 mAb6 or with Matrin-3 antibody. CSK and NM were isolated from the same nuclear fractions. The amounts used in each lane corresponded to 4 μ g (CSK) and 10 μ g (matrix) of the isolated nuclear fraction.

are comparable to the fast population relative size (46%) and recovery rate ($t_{1/2} = 0.27$ s) estimated from the FRAP curve (Figure 3, B and C).

The role of the NM in regulating FGFR1-EGFP's mobility was next examined. The release of FGFR1-EGFP from the NM by Cyt D (see Figures 2B and 4D) was accompanied by a fourfold reduction in the immobile FGFR1-EGFP population (Figure 3D), indicating that the immobile population represents NM-bound FGFR1-EGFP. Actin depolymerization produced a small, but significant ($p < 0.001$, increase in both the fast (1.3-fold) and slow (1.4-fold) populations. In addition, the $t_{1/2}$ of the slow population increased twofold with actin depolymerization (Figure 3D).

Lactacystin-treated cells also displayed a decrease in the immobile FGFR1-EGFP population (Figure 3D), consistent with the reduction of matrix-associated FGFR1-EGFP (Figure 1). This reduction in the immobile FGFR1-EGFP was accompanied by a threefold increase in the slow FGFR1-EGFP population and a twofold decrease in the fast population. Additionally, lactacystin treatment significantly increased the recovery half-time of both the fast and slow FGFR1-EGFP populations by seven- and sixfold, respectively (Figure 3D).

Transcription State Influences FGFR1 Mobility

FGFR1 nuclear accumulation and association with transcriptional are both stimulated by cAMP. These events are accompanied by the nuclear coaccumulation of FGF-2, FGFR1 binding to the transcription coactivator CBP, and transcriptional upregulation (Stachowiak *et al.*, 2003b; Hu *et al.*, 2004; Fang *et al.*, 2005).

First, we examined the effect of dBcAMP on FGFR1-EGFP nuclear dynamics. The overall shape of the FRAP curve was profoundly changed by dBcAMP because of the loss of the immobile population and an overall decrease in the FGFR1-EGFP recovery rate (Figure 4A). Two-exponential analyses revealed that dBcAMP treatment increased the slow FGFR1-EGFP population (over fourfold), reduced the fast population (2.5-fold), and nearly eliminated the immobile population (Figure 4A). The recovery half-time of the slow population was reduced (6-fold) indicating that dBcAMP causes FGFR1 to participate in molecular events that reduce its movement.

In contrast to the dBcAMP, the transcriptional inhibitors (Act D or DRB) both accelerated the FGFR1-EGFP recovery

rate (Figure 4B; $p < 0.05$). Two-exponential analyses revealed that both Act D and DRB increased the fast population (twofold) and reduced the slow population of FGFR1-EGFP (one-third relative to nontreated cells; not shown), the latter effects being opposite to those of dBcAMP. Moreover, Act D antagonized the effect of the transcription activator dBcAMP on FGFR1-EGFP (Figure 4B). Thus, transcriptional activation, which induced an FGFR1 shift from the NM to chromatin, coincided with depletion of the immobile FGFR1 population, an increase in the slow population and a reduction in the rate of its movement. In contrast, transcriptional inhibition increased the rate of FGFR1 movement.

Nuclear FGFR1 Mobility Is Affected by Its Binding Partner CBP

Transcriptional activation by nuclear FGFR1 is synergistically enhanced by cotransfected CBP and blocked by antisense CBP RNA. CBP transfection increased the TE671 cell content of CBP two- to threefold, whereas antisense RNA depleted endogenous CBP by ~80% (Fang *et al.*, 2005). These changes in CBP content were confirmed in the present study (not shown). FRAP analysis revealed that cotransfection with CBP abolished FGFR1's immobile population, reduced the fast population, and increased the slow mobile population (Figure 4C). CBP also increased (more than fivefold) FGFR1's slow nuclear recovery half-time. These effects of CBP were similar to changes produced by cAMP (Figure 4A).

To determine whether endogenous CBP influences nuclear FGFR1-EGFP mobility, CBP was depleted by cotransfection of a plasmid expressing antisense CBP RNA. CBP depletion decreased the slow mobile FGFR1-EGFP population and produced an equivalent increase in the fast population (Figure 4C). These effects were opposite to the effects produced by overexpressed CBP and further support CBP's role in generating FGFR1's slow, chromatin-binding population.

To determine whether association with the FGFR1-binding N-terminal domain of CBP was sufficient to change FGFR1 mobility, we cotransfected FGFR1-EGFP either with the FGFR1 binding fragment of CBP (amino acids 451-689), or a C-terminal region of CBP that does not bind FGFR1 (amino acids 1678-2441; Fang *et al.*, 2005). Cotransfection of CBP (451-689) increased the $t_{1/2}$ (3.6-fold) of the slow FGFR1 population and its relative size, while significantly decreasing both the immobile and fast FGFR1 populations (Table 1).

Table 1. Nuclear FGFR1 movement is affected by its binding partners CBP and RSK1

Transfected plasmids	Fast half-time	Slow half-time	Fast mobility (%)	Slow mobility (%)
R1-EGFP	0.29 ± 0.02	24.41 ± 3.48	46.14 ± 1.57	17.68 ± 1.52
R1-EGFP + CBP	0.55 ± 0.26	133.3 ± 14.91*	25.35 ± 2.22*	75.12 ± 5.53*
R1-EGFP + CBP (451-689)	0.44 ± 0.02	87.45 ± 12.56*	35.25 ± 2.03*	49.02 ± 2.0*
R1-EGFP + CBP (1678-2441)	0.36 ± 0.10	33.71 ± 0.65	38.96 ± 1.78*	0.92 ± 2.89*
R1-EGFP + RSK1	0.41 ± 0.05	36.83 ± 3.96*	48.17 ± 1.24	18.39 ± 1.25
R1(TK-)-EGFP	3.395 ± 0.30*	ND*	27.32 ± 0.75*	ND*
R1(TK-)-EGFP + CBP	0.21 ± 0.02*	19.61 ± 2.41*	47.36 ± 2.27*	6.12 ± 0.91*

Results of FRAP measurements; half-times ($t_{1/2}$) are expressed in seconds. FGFR1(R1)-EGFP was cotransfected with RSK1, CBP-expressing plasmid or with plasmids expressing FGFR1-binding (aa 451-689) or nonbinding (aa 1678-2441) fragments of the CBP. Results for CBP are shown also in Figure 4C and are listed for comparison. Total amount of cotransfected DNA was kept constant with pcDNA3.1. Transcriptionally inactive FGFR1(TK-) fused to EGFP [R1(TK-)-EGFP] was co-transfected with pcDNA3.1, CBP, or RSK1. CBP significantly increased R1-EGFP's slow population and recovery half-time, while decreasing the immobile and fast populations (* $p < 0.001$). The slow recovery half-time was significantly increased in the presence of RSK1 (* $p < 0.001$), whereas no significant effect could be seen in the mobile or immobile population ($n \geq 24$). The recovery half-time and mobility of R1(TK-)-EGFP was significantly altered in the presence of CBP (* $p < 0.001$), but to a lesser extent than that observed with R1-EGFP ($n \geq 14$).

The overall shift in FGFR1's mobility induced by the CBP fragment (451-689) were similar to those observed with full-length CBP (compared in Table 1), albeit the changes induced by the CBP fragment were smaller. In contrast, expression of the C-terminal CBP fragment (1678-2441) significantly increased (1.8-fold) the immobile population and markedly depleted the FGFR1 slow population (Table 1). The recovery half-time of the residual slow population was only slightly reduced (1.4-fold) by CBP (1678-2441).

Incubation of cells with dBcAMP or transfection with CBP increased FGFR1 association with chromatin, while reducing the receptor population associated with the NM (Figure 4D). These effects are consistent with the FGFR1-EGFP mobility shifts observed in the presence of dBcAMP and CBP (i.e., depletion of the immobile population, relative increase in the slow population and its diminished velocity; Figure 4, A and C). Together these findings indicate that cAMP shifts FGFR1-EGFP from the NM to chromatin, similar to that previously observed with endogenous FGFR1 (Stachowiak *et al.*, 1996b), and furthermore an effect that may involve the FGFR1-binding partner and transcription coactivator CBP.

The FGFR1-binding Transcriptional Coregulator 23 kDa FGF-2 Modulates FGFR1's Nuclear Dynamics

FGFR1 can effectively bind to LMW 18-kDa FGF-2 (found both intracellularly and extracellularly), as well as the N-terminally extended predominantly nuclear HMW FGF-2 (21, 22.5, and 23 kDa) isoforms (Sheng *et al.*, 2005). Although HMW-FGF-2 is an effective coactivator of nuclear FGFR1 stimulated genes, exogenous LMW FGF-2 is not (Stachowiak *et al.*, 2003b). Furthermore, 23-kDa HMW FGF-2 increased the nuclear accumulation of cotransfected FGFR1 in TE671 cells, whereas LMW FGF-2 did not (Figure 1, B and C).

To determine whether FGF-2 influences FGFR1's mobility, the effects of both HMW (23 kDa) FGF-2 and LMW (18 kDa) FGF-2 were examined. Cotransfection of HMW FGF-2 reduced (more than fivefold) the immobile FGFR1 population (Figure 5A), whereas the slow population significantly increased in relative size (more than twofold), as well as in the recovery half-time (10-fold). LMW FGF-2 also reduced (less than twofold) FGFR1-EGFP's immobile population; however, the effect of LMW-FGF-2 was significantly smaller than that observed with HMW FGF-2. Moreover, in contrast to HMW FGF-2, LMW FGF-2 reduced the slow population and had no significant effect on FGFR1's recovery rate (Figure 5A). Both FGFs slightly increased (by one-third) the size of the fast FGFR1 population.

To determine if the differential effects of the two FGF-2 isoforms on FGFR1's mobility may reflect differences in their interaction with the receptor, coimmunoprecipitation experiments were carried out. HMW (23 kDa) or LMW (18 kDa) FGF-2 tagged with the flag epitope were cotransfected with the receptor. Subcellular fractions (membrane-mitosomal, soluble-cytosolic, and nuclear) were isolated and were analyzed on Western blots with anti-Flag Ab (Figure 5B, top row). In addition, the isolated fractions were treated with the C-terminal FGFR1 Ab, and the immunoprecipitates were analyzed on Western blots with anti-Flag or FGFR1 mAb6 (Figure 5B). Both transfected HMW and LMW FGF-2 were detected in all three fractions, although HMW FGF-2 was relatively more abundant in the nuclear fraction than LMW (Figure 5B, top row). Transfected FGFR1 was efficiently immunoprecipitated from the mitosomal, cytosolic, and nuclear frac-

tions of cells cotransfected with 18- or 23-kDa FGF-2 (bottom row) using the FGFR1 C-terminal Ab. In 23-kDa FGF-2-cotransfected cells, the HMW ligand coprecipitated with FGFR1 from all three fractions (middle row), whereas LMW FGF-2 only coimmunoprecipitated with FGFR1 in the mitosomal fraction.

The intranuclear interaction between FGFR1 and 23-kDa FGF-2 (and lack of a 18-kDa FGF-2-FGFR1 interaction) was verified by FRET analyses (Figure 5C). In cells transfected with FGFR1-CFP and 23-kDa FGF-2-YFP, bleaching of the fused YFP acceptor increased the fluorescence emitted by the FGFR1-CFP donor (as illustrated by two sequential HMW-FGF-2 bleachings of adjacent regions in the cell nucleus; Figure 5C, top). This FRET signal was detected in the nucleolus and nucleus. In contrast, we observed no effect of acceptor (FGFR1-YFP) bleaching in the nucleus on the fluorescence emission by 18-kDa FGF-2-CFP donor (Figure 5C, bottom left). No detectable FRET occurred between cotransfected nuclear 18-kDa FGF-2-CFP and 23-kDa FGF-2-YFP (Figure 5C, bottom right) or between FGFR1-CFP and non-fused YFP (not shown). Thus, although HMW (23 kDa) FGF-2 serves as a ligand for nuclear FGFR1, LMW (18 kDa) FGF-2 does not interact with nuclear FGFR1.

FGFR1 Tyrosine Kinase Domain Influences Nuclear Receptor Dynamics

The FGFR1(TK-) mutant binds to CBP, but lacks the tyrosine kinase (TK) domain that is essential to activate transcription (Stachowiak *et al.*, 2003b; Fang *et al.*, 2005) and to bind to many other proteins (Hu *et al.*, 2004; Fang *et al.*, 2005). Transfected FGFR1(TK-)-EGFP displayed an immobile nuclear population that was twice as large (72.68%; Table 1) as the full-length FGFR1-EGFP (36.18%). Two-exponential analyses revealed elimination of the slow population and reduction (twofold) of the fast population (Table 1). The fast recovery half-time of FGFR1(TK-)-EGFP was significantly higher than that of FGFR1-EGFP. Thus, the TK domain is essential to maintain the slow kinetic population, the population that appears to be actively involved in transcription.

A slow FGFR1(TK-)-EGFP population (6%; $t_{1/2} = 19.4$ s) was detectable only when FGFR1(TK-)-EGFP was cotransfected with CBP (Table 1), and also CBP increased the fast population of FGFR1(TK-)-EGFP (from 27 to 47%). Thus, although CBP is sufficient to release FGFR1(TK-)-EGFP from the immobile pool, the slow FGFR1(TK-)-EGFP pool was not generated as efficiently as that observed with FGFR1-EGFP. In addition, CBP reduced (>10-fold) the $t_{1/2}$ of FGFR1(TK-)-EGFP's fast population, an effect not observed with FGFR1-EGFP (Table 1).

The above results indicated that the interaction of FGFR1's TK domain with other proteins is important for generating the slow kinetic FGFR1-EGFP population. One candidate protein was pp90RSK1, which binds to the TK containing region of FGFR1 (Hu *et al.*, 2004; Fang *et al.*, 2005; Dunham-Ems *et al.*, 2006). Cotransfection of RSK1 (which mimics two- to threefold increase in nuclear RSK1 levels observed during cell stimulation; Hu *et al.*, 2004) with FGFR1-EGFP had no significant effect on the relative abundance of nuclear FGFR1's kinetic pools, but did significantly increase (1.5-fold) the recovery half-time of the slow population (Table 1). Thus, FGFR1's interaction with RSK1 retards FGFR1's movement, possibly by increasing the length of time that FGFR1 is associated with target genes. However, unlike cAMP, CBP, or 23-kDa FGF-2, RSK1 did *not* increase the relative size of the slow FGFR1 population.

Figure 5. Transcriptional coregulator HMW FGF-2 modulates FGFR1's dynamics and interacts with nuclear FGFR1. (A) Effect of FGF-2 on FGFR1 mobility in the nucleus. FGFR1 (R1)-EGFP was cotransfected with control vector or with 18- or 23-kDa FGF-2-expressing plasmids. Eighteen-kilodalton FGF-2 significantly increased the relative size of the fast population and decreased the slow and immobile populations ($p < 0.001$), and 23-kDa FGF-2 significantly increased the relative sizes of the fast and slow mobile populations and decreased the immobile population ($p < 0.001$). The slow recovery half-time was significantly ($p < 0.001$) increased in the presence of 23-kDa FGF-2 but not by 18-kDa FGF-2 ($n \geq 14$). (B) Coimmunoprecipitation of FGFR1 with FGF-2. TE671 were transfected with FGFR1 (R1) and with pcDNA 3.1 vector (lanes 1, 3, and 5) or plasmid expressing with flag-tagged 18- or 23-kDa FGF-2 (lanes 2, 4, and 6). Top row, immunoblotting (IB) of transfected FGF-2

DISCUSSION

The present study further substantiates the nuclear localization and function of FGFR1 in live cells. The FRAP experiments show that the nuclear and cytoplasmic compartments contain distinct FGFR1 kinetic populations. In the nucleus the overall movement rate of the FGFR1 is faster, whereas the mobile FGFR1 fraction is smaller than in the cytoplasm. The mobility of nuclear FGFR1 reflects FGFR1's association with subnuclear compartments, nuclear proteins as well as its transcriptional function. Three nuclear FGFR1 populations can be distinguished: 1) a fast mobile population whose rate is indistinguishable from the cytoplasmic fast FGFR1 population, 2) a slow mobile population (two times slower than the slow population in the cytoplasm), reflecting chromatin-bound FGFR1, and 3) an immobile population representing the NM-bound receptor. FGFR1's association with the NM involves its interaction with actin, indicated by the release of NM-bound FGFR1 by Cyt D and by the coimmunoprecipitation of these two proteins. The release of FGFR1-EGFP from the NM by Cyt D (Figures 2B and 4D) was accompanied by a fourfold depletion of the immobile FGFR1 population (Figure 3D) and a twofold reduction in the movement rate of the slow FGFR1 population. Thus, FGFR1's interaction with actin appears to have a dual role in the control of FGFR mobility involving 1) maintenance of the FGFR1 immobile population and 2) facilitation of the movement of the mobile FGFR1 molecules. Whether FGFR1 binds directly or indirectly to actin filaments is unknown at present.

Identification of the FGFR1 immobile population as NM-bound molecules was further corroborated by the cAMP- or CBP-induced FGFR1 redistribution from the NM to chromatin. This redistribution also was accompanied by depletion of the immobile receptor and an increase in the slow mobility FGFR1 populations. Additionally, proteasome inhibition reduced matrix-associated FGFR1-EGFP (Figure 2C) and markedly decreased (threefold) the immobile FGFR1-EGFP population while increasing the slow and fast FGFR1 populations (Figure 3D). Thus, proteasome activity promotes FGFR1's association with the NM. This result is in contrast to the estrogen receptor- α (Stenoien *et al.*, 2001b), whose binding to the NM was stabilized by proteasome inhibition. A plausible explanation involves proteasome-mediated degradation of factors that mobilize FGFR1, thereby preventing FGFR1's release from the NM. Proteasome inhibition also significantly increased the recovery half-time of FGFR1, similar to the findings with other nuclear receptors, which may reflect formation of large protein complexes (Stenoien *et al.*,

2001b; Deroo *et al.*, 2002). Thus, proteasomal activity provides effective mechanisms for the generation of different mobility pools and subsequently the different subnuclear compartmentalization of FGFR1.

Two distinct mobile populations, fast and slow, have been identified for several chromatin-binding proteins (Becker *et al.*, 2002; Phair *et al.*, 2004). The fast was proposed to represent freely diffusing proteins that undergo rapid nonspecific intermolecular collisions while the slower fractions represent molecules engaged in specific binding events. Chromatin binding reduces the recovery rate of ligand-bound steroid receptors, which become temporarily immobilized on target genes (Stenoien *et al.*, 2001b; Phair *et al.*, 2004) and of RNA polymerases I and II upon transcriptional engagement (Becker *et al.*, 2002; Dundr *et al.*, 2002; Kimura *et al.*, 2002). The mean residence time of chromatin-binding proteins is 2–20s, with generally no significant immobile population (Phair *et al.*, 2004). The recovery half-times of both the fast and slow FGFR1 populations fall into this range. Approximately 35% of nuclear FGFR1 is immobile which is not as highly mobile as transcription regulators and yet not as immobile as the core histones (Phair *et al.*, 2004). Fang *et al.* (2005) proposed that FGFR1 acts as a common “gate opening factor” that promotes transcriptional activation by releasing CBP from RSK1-mediated inhibition. As shown here, this function is associated with FGFR1 segregation into transcriptionally active nuclear microdomains. Moreover, we observed an increase in the overall mobile population and reduced rate of FGFR1 movement with factors that activate FGFR1-mediated transcription (cAMP, CBP, and HMW-FGF-2), and the opposite changes were observed with transcriptional inhibitors, antisense CBP, and transcriptionally inactive FGFR1(TK–).

Our studies shed light on the roles of FGFR1 ligands in biology and the function FGFR1. Even though both FGFs can potentially bind to FGFR1, we show, for the first time, that nuclear FGFR1 interacts specifically with HMW (23 kDa) FGF-2, but not with LMW (18 kDa) FGF-2. Furthermore, LMW FGF-2, which does not bind or act via nuclear FGFR1, failed to reproduce the mobility and nuclear accumulation effects of HMW FGF-2 which coactivates transcription with nuclear FGFR1. Thus, FGFR1 and LMW FGF-2 may be present in different nuclear functional domains or, alternatively, LMW FGF-2 may be engaged in binding with other nuclear proteins that may prevent its interaction with the nuclear FGFR1. The FGFR1–HMW FGF-2 interaction induces generation of fast and slow mobile FGFR1 molecules. The slowing of FGFR1 molecules by HMW FGF-2 may reflect FGFR1 binding to FGF-2 and association with gene promoters, which is supported by other studies (Fang *et al.*, 2005).

CBP has been shown to transduce nuclear FGFR1 stimulation. Both proteins associate with promoters in a manner that correlates with transcriptional activation by cAMP and protein kinase A pathways or HMW FGF-2 (Fang *et al.*, 2005). CBP-induced depletion of the immobile FGFR1 population increased slow population, and its reduced average rate of movement was similar to the changes produced by stimuli (cAMP, HMW FGF-2) that engage FGFR1 in transcriptional stimulation. These changes appear to reflect FGFR1 binding to the N-terminal region of the CBP based on the similar effects produced by the full-length CBP and N-terminal CBP fragment that binds FGFR1 but not by the FGFR1 nonbinding C-terminal CBP region. The opposite changes in FGFR1 mobility induced by C-terminal region could potentially reflect a competition between the CBP fragment and endogenous transcription coactivators. Thus,

Figure 5 (cont). with anti-flag Ab in microsomal (lanes 1 and 2), cytosolic (lanes 3 and 4), and nuclear (lanes 5 and 6) fractions. Middle and bottom rows, FGFR1 was immunoprecipitated (IP) with C-terminal FGFR1 Ab (R1ct). The precipitates were immunoblotted with N-terminal FGFR1 *mcAb6* or with anti-flag Ab. Twenty-three kilodalton FGF-2 was coprecipitated with FGFR1 from all three fractions. In contrast, 18-kDa FGF-2 was coprecipitated with FGFR1 only from the microsomal fraction. (C) Interaction of FGFR1 with FGF-2 in the nucleus: FRET analysis. TE671 cells were transfected with FGFR1-CFP + 23-kDa FGF-2-YFP (top panels) or with FGFR1-YFP + 18-kDa FGF-2-CFP (bottom left panel). In addition, 18-kDa FGF-2-CFP was cotransfected with 23-kDa FGF-2-YFP (bottom right panel). FRET was measured using acceptor bleaching (*Materials and Methods*). Similar results were obtained in 3–5 cells per treatment in three independent transfection experiments. Scale bar, 25 μ m. Color scale of FRET efficiency: 0.500 = 50%. The efficiency of energy transfer between nuclear 23-kDa FGF-2 and FGFR1 was 35–40%.

Figure 6. Protein movement and stochastic collisions regulate FGFR1-induced transcription. There are three kinetic FGFR1 populations. Nuclear matrix-bound FGFR1 remains relatively immobile and may represent “stored” FGFR1 or receptor involved in other than transcription functions. Fast mobile population represents transcriptionally inactive FGFR1 would randomly sample chromatin sites via rapidly occurring collisions. A slow mobile FGFR1 population forms upon FGFR1 interactions with HMW FGF-2 and CBP that target FGFR1 to chromatin and form transcriptionally active chromatin sites. FGFR1 and CBP coassociation with gene promoter DNA was shown by chromatin immunoprecipitation (Fang *et al.*, 2005).

the present study lends further support for the role of CBP as a FGFR1-binding transcription activating partner that transiently immobilizes FGFR1 to chromatin in live cells. CBP also retarded the movement of the tyrosine kinase deleted receptor, but not to the extent observed with full-length FGFR1 and, therefore, additional proteins that bind to FGFR1 tyrosine kinase domain may be involved in regulating FGFR1 mobility and distribution. One such factor could be RSK1, which binds to the FGFR1 tyrosine kinase region and accumulates in the nucleus along with FGFR1 during cAMP transcription stimulation (Hu *et al.*, 2004; Fang *et al.*, 2005). The results of RSK1 transfection indicate that the increased FGFR1–RSK1 interaction retards the movement of mobile FGFR1 molecules, possibly by increasing the length of time that FGFR1 is associated with the target genes. However, unlike cAMP, CBP, or HMW FGF-2, RSK1 did not increase the relative size of the slow FGFR1 population. Further investigations should determine what tyrosine kinase-binding nuclear factors are involved in the generation of the FGFR1 slow, chromatin-binding population and transcriptionally active receptor.

The results of our present investigation support a general gene activation mechanism, where protein movement and collisions with other proteins and nuclear structures governs formation of the macromolecular complexes involved in transcription. A plausible model of gene regulation by nuclear FGFR1 is summarized on Figure 6. FGFR1 that is not engaged in transcription associates with the NM and thus is relatively immobile. Transcription activating cAMP releases FGFR1 from the NM, thereby generating mobile receptor populations via processes that involve FGFR1’s interaction with 23-kDa FGF-2 and CBP. A fast oscillating FGFR1 population is generated that engages in rapid “nonproductive” molecular collisions and chromatin scanning. CBP binding generates a slow FGFR1 mobile population that may undergo further binding events with other proteins and promoters forming transcriptionally productive complexes (as shown in Fang *et al.*, 2005) with further reduced oscillation rates. The increased chromatin residence time of such complexes caused by proteins that bind to FGFR1’s TK domain (i.e., RSK1) may allow transcription to be initiated and/or carried out.

Act D and DRB inhibit transcription via different mechanisms. Act D inhibits RNA Pol II C dephosphorylation, necessary for the initiation of transcription (Dubois *et al.*, 1994) and immobilization of the RNA Pol II complex. In contrast, DRB blocks RNA Pol II phosphorylation and thus interferes with reentry into elongation after pausing and generation of nascent transcripts (Marshall and Price, 1992; Dubois *et al.*, 1994). Thus, depletion of slow FGFR1 population by both Act D and DRB suggests that the slow population could represent FGFR1 molecules engaged in transcription initiation as well as molecules engaged in transcript elongation. These FGFR1 functions have previously been suggested because of FGFR1-induced stimulation of RNA Pol II recruitment to gene promoters (Fang *et al.*, 2005) and colocalization with the hyperphosphorylated CTD-phosphorylated form of RNA Pol II (Somanathan *et al.*, 2003).

Changes in FGFR1 nuclear dynamics observed during transcriptional activation are generally consistent with those of nuclear steroid receptors (McNally *et al.*, 2000; Becker *et al.*, 2002), long established transcriptional factors. Features that separate FGFR1 from the steroid receptors (Stenoien *et al.*, 2001a,b) include FGFR1 immobilization in the NM and mobilization accompanying transcription stimulation. This process provides an effective mechanism for storing FGFR1 in the nucleus, thus limiting its spatial activity to sites where FGFR1 is transiently mobilized. This mechanism could allow FGFR1 to coordinately activate genes in many chromatin loci as proposed by the “feed forward and gate” regulation of multigene programs.

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