90-kDa Ribosomal S6 Kinase Is a Direct Target for the Nuclear Fibroblast Growth Factor Receptor 1 (FGFR1)

ROLE IN FGFR1 SIGNALING*

Yafang Hu‡, Xiaohong Fang, Star M. Dunham§, Claudia Prada, Ewa K. Stachowiak, and Michal K. Stachowiak¶

Received for publication, October 9, 2003, and in revised form, April 26, 2004 Published, JBC Papers in Press, April 26, 2004 DOI 10.1074/jbc.M311144200

Fibroblast growth factor receptor 1 (FGFR1) is a transmembrane protein capable of transducing stimulation by secreted FGFs. In addition, newly synthesized FGFR1 enters the nucleus in response to cellular stimulation and during development. Nuclear FGFR1 can transactivate CRE (cAMP responsive element), activate CRE-binding protein (CREB)-binding protein (CBP) and gene activities causing cellular growth and differentiation. Here, a yeast two-hybrid assay was performed to identify FGFR1-binding proteins and the mechanism of nuclear FGFR1 action. Ten FGFR1-binding proteins were identified. Among the proteins detected with the intracellular FGFR1 domain was a 90-kDa ribosomal S6 kinase (RSK1), a regulator of CREB, CBP, and histone phosphorylation. FGFR1 bound to the N-terminal region of RSK1. The FGFR1-RSK1 interaction was confirmed by coimmunoprecipitation and colocalization in the nucleus and cytoplasm of mammalian cells. Predominantly nuclear FGFR1-RSK1 interaction was observed in the rat brain during neurogenesis and in cAMP-stimulated cultured neural cells. In TE671 cells, transfected FGFR1 colocalized and communoprecipitated, almost exclusively, with nuclear RSK1. Nuclear RSK1 kinase activity and RSK1 activation of CREB were enhanced by transfected FGFR1. In contrast, kinase-deleted FGFR1 (TK–), which did not bind to RSK1 failed to stimulate nuclear RSK1 activity or RSK1 activation of CREB. Kinase inactive FGFR1 (K514A) bound effectively to nuclear RSK1, but it failed to stimulate RSK1. Thus, active FGFR1 kinase regulates the functions of nuclear RSK1. The interaction of nuclear FGFR1 with pluripotent RSK1 offers a new mechanism through which FGFR1 may control fundamental cellular processes.

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adapts of adrenal medullary cells (12, 13, 20), which were prevented by transfection of the cytoplasmic/nuclear dominant negative FGFR1(TK−), or its exclusively nuclear derivative in which the signal peptide (SP) was replaced by an NLS. Cellular growth and functional adaptations were induced by transfection of the active wild type cytoplasmic/nuclear FGFR1 or the nonmembrane/nuclear FGFR1 mutant (11, 13, 20, 21). However, these events were not reproduced by the membrane-sequestered mutant or by stimulation or inhibition of surface FGFR (13, 20, 21).

FGFR1 accumulates specifically within nuclear matrix attached (0.5–2 μm) speckle domains rich in splicing factors, phosphorylated histones, and phosphorylated RNA polymerase II (13, 14, 21). In these speckles, FGFR1 associates specifically with sites of RNA transcription (14). Nuclear FGFR1 was shown to activate a number of endogenous genes and their promoters and to mediate their activation by extracellular signals and their second messengers (13, 20, 21).

Recent studies have shown that nuclear FGFR1 is a powerful activator of CREB-binding protein (CBP) and can stimulate CREB phosphorylation (21). CREB can be directly phosphorylated and activated by a number of kinases including cAMP-activated protein kinase A, by Ca2+/calmodulin-dependent protein kinase and 90-kDa ribosomal S6 kinases (RSK) (22). Angiotensin II, depolarization, or phorbol 12-myristate 13-acetate activation of CREB-mediated transcription were all blocked by dominant negative FGFR1, whereas cAMP induced activation was not (23), which indicated that nuclear FGFR1 activates CREB through a CREB, which is not PKA. One candidate CREB kinase that could potentially be targeted of nuclear FGFR1 may be produced in cooperation with RSK1. The present study was undertaken to identify proteins functions of nuclear FGFR1, which include activation of CREB may be produced in cooperation with RSK1.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid Assay**—The MATCHMAKER two-hybrid system 3 (Clontech, Palo Alto, CA) was used to screen a human fetal brain cDNA library in pACT2 (Clontech, Palo Alto, CA) according to the manufacturers protocol. The cDNA fragment coding the C-terminal portion of FGFR1 (CR1; amino acids, aa 399–822) was cloned in-frame with the Gal4 DNA-binding domain (BD) into pGBK9 (6) and was provided by Dr. Pam Maher (Scripps Research Institute, La Jolla, CA). The cDNA fragment containing the N-terminal portion of FGFR1 (NR1, aa 1–401) was fused with the Gal4 BD in the high expression vector pGBK7-7 (Clontech). pGBK9-CR1 and pGBK7-7-NRII were transformed into the AH109 yeast strain (genotype: Mata, trpl-901, leu2-3, 112, ura3-52, his3-200 gal4Δ, gal80Δ, lys2Δ, GAL1Δ-AAL, GAL1Δ-TATA-HIS3, GAL2Δ-AAL, GAL2Δ-TATA-ADH2, URA3::MEL1Δ-AAL, MEL1Δ-TATA-lacZ). Expression of BD-CR1 and BD-NRII fusion proteins in strain AH109 were verified by Western blotting with the anti-Gal4 BD antibody (Clontech). The cDNA library was transfected into AH109 strains expressing CR1 or NRII bait plasmids. The transformants were selected for the expression of HIS3, ADE2, and MEL1, by growing on synthetic dropout (SD) medium (SD/-Trp/-Leu/-His/-Ade/X-a-gal) with the drop of trypotphan, leucine, histidine, and adenine and X-a-gal. cDNA plasmids were isolated from the positive colonies, sequenced for inserts, and introduced into the Y187 strain (genotype: Mata, ura3-52, his3-200, ade2-101, trpl-901, leu2-3, 112, gal4Δ, met +, gal80Δ, URA3::MEL1Δ-AAL, GAL1Δ-TATA-lacZ). Y187 strains containing individual cDNA plasmids were mated separately with AH109 cells expressing BD baits, BD, or HD laminin C.

**Constructions and Mutagenesis**—One of the isolated cDNA inserts contained a partial cDNA of RSK1 (aa 36–735). To obtain the cDNA encoding full-length RSK1, polymerase chain reaction was utilized to amplify the 5’ coding sequence from the 5’ gene fragment of RSK1 (Incyte Genomics Inc.). The PCR fragment (aa 1–70) was generated using the 5’ primer, 5′-GGGCGGAATTCTGAGAGCTCGAATG-3′, and 3’ primer, 5′-ACCTTGAGGAGCTCGAAATG-3′. The fragment was digested with EcoRI/Sacl and ligated to the 5’ end of the partial RSK1 cDNA in pACT2. The cDNA encoding full-length of RSK1 was excised and inserted in-frame with AD in yeast pGAD7 vector (Clontech), as an FLAG tag in the mammalian expression vector pCMV-Tag2 (Strategene, Palo Alto, CA).

Four C-terminal deletions of RSK1 were generated by PCR using the same 5′-AGTGAAATTCAGGATGACCGGCTTCCC-3′ and variable 3′ primers: 5′-CAGCTCGAGGTTGCTGCTGTCGTA-3′ for the KC1 mutant (aa 36–850); 5′-CAGCTCGAGGACGATAGCTGCGGACGCTTCA-3′ for the KC2 mutant (aa 36–550); 5′-CAGCTCGAGGACGATAGCTGCGGACGCTTCA-3′ for the KC3 mutant (aa 36–450); and 5′-CAGCTCGAGG-TAGAAGGGTCTCATCAGG-3′ for the KC4 mutant (aa 36–350). Other RSK1 mutants were generated by introducing deletions into RSK1 or KC4 in pGAD7 with restriction enzymes. The KC5 contains aa 36–325, KC6 contains aa 36–218, KN1 contains aa 43–350, KN2 contains aa 65–350, KN3 contains aa 71–350, and KN4 contains aa 219–350. A Galactosidase Quantitative Assay—The assay was performed according to the Clontech protocol. AH109 yeast cells were cotransformed with the Gal4 BD in the high expression vector pCMV-Tag2 (Strategene, Palo Alto, CA).
FGFR4 (IYASCSGLALAVLLGLYRGG) as described previously (13). Kinase inactive FGFR1 mutant (FGFR1/K514A) was generated by replacing lysine 514 with alanine in pcDNA3.1FGFR1 using the Strategene QuickChange™ site-directed mutagenesis kit. The primers used were: gacaaagtctgggtgattgaagcaag and cttgactaattaacgccagcacagcctttgt.

Cultures of Mammalian Cells and Transfections—Human TE671 cells (express low levels of endogenous FGFR1) were grown in Dulbecco’s modified Eagle’s medium as described previously (15). Fetal neural stem cell line, HUVEC-NSC, derived from the nonhuman primate population of the human umbilical cord blood (35) was obtained from Dr. Krystyna Domanska-Janik (Polish Academy of Science, Warsaw, Poland). All culture media were supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen) and maintained at 37 °C in 5% CO2 in a humidified incubator. Cells were kept in serum-free medium 24 h before drug treatment or transfection until the end of experiment.

TE671 were transfected using calcium-phosphate DNA precipitation as previously described (20). Eighty percent confluent TE671, plated 1 day prior, were transfected with 1 μg of pCMV-FlagRSK1, pcDNA3.1-FGFR1 or control pcMV-FLAG or pcDNA3.1 vectors. After 48 h, cells were harvested and lysed in lysis buffer (pH 7.4, phosphate-buffered saline containing 1% Triton X-100, rich complete protease inhibitor mixture, 1 mM phenylmethylsulfonyl fluoride). Cell Fractionation and Characterization of Subcellular Fractions—Nuclear and cytoplasmatic fractions were isolated and characterized as previously described (12, 21). Briefly, cells were lysed in buffer containing 0.25 M NaCl, 10 μM protease inhibitor mixture (Sigma), 25 mM NaF, 2 mM dithiothreitol, 1 μM phenylmethylsulfonyl fluoride, 0.1% Nonidet P-40 in 10 mM Hepes, pH 7.5, 10 mM KCl, 0.1 mM EDTA, and 0.1 mM EGTA. The lysates were then centrifuged at 8,000 rpm for 1 min and the supernatant representing the cytoplasmic fraction with cytoskeletal elements was collected after centrifugation. The pellets were washed three times and then sonicated in 1% Triton X-100 buffer, pH 7.4, containing 50 mM Hepes, 50 mM NaCl, 5 mM EDTA, and protease inhibitors. The isolated nuclei contained 90% of the total trichloroacetic acid-precipitable DNA and were characterized as previously described (12, 13, 20, 21, 36, 37). Nuclear FGFR1 co-purified with typical nuclear nucleosomes such as (i) histones H3/H4 detected on Coomasie-stained gels (established marker of the nuclear fraction (38, 39)), (ii) CREB and CBP (13), or (iii) proteins engineered to accumulate specifically in the nucleus by insertion of the nuclear localization signals (13, 20). Phase-contrast microscopy showed little contamination of the isolated nuclei by cytoplasmatic membranes and organelles. The purity of the isolated nuclei was confirmed also by biochemical assays of marker enzymes as previously described (12, 13, 21, 36, 37). The nuclei clearly contained less then 5% of the total cellular activity of 5′-nucleotidase (plasma membrane marker) and less than 2% of total activity of acid phosphatase (lysosomal marker). In FGFR1-transfected TE671 cells, in which the cell surface proteins were biotinylated with cell impermeable NHS-sulfobiotin, the biotinylated FGFR1 was detected in the extranuclear fraction but not in the nuclear fraction as shown previously (13, 14). Thus, the appearance of FGFR1 in the nucleus is not the result of contamination by plasma or cytoplasmic membrane-associated FGFR1 and the nuclear receptor was not derived from the cell surface (13, 14).

Animals—Prenatal (E23) Spraque-Dawley rats were used according to IACUC approved protocol. Nuclear and cytoplasmic fractions were isolated as described above and characterized as previously described (12, 13, 20, 21, 36, 37).

Antibodies, Immunoprecipitation, and Immunoblotting—Specific rabbit polyclonal RSK1 antibody against the C-terminal RSK1 region and sheep polyclonal antibody against phospho-Ser364 (rat numbering) (see Ref. 40 for description) were purchased from Upstate Biotechnology (Lake Placid, NY). Polyclonal rabbit antibody against C-terminal FGFR1 was from Santa Cruz Biotechnology (Santa Cruz, CA), and monoclonal anti-FLAG tag was from Sigma. Affinity purified monoclonal FGFR1 (McAb6) and polyclonal C-terminal FGFR1 were described by Hanneken et al. (41). Their specificity was confirmed in several reports (12, 13, 15, 18, 20, 36, 37).

Protein content was then determined using the Bio-Rad assay. For Western immunoblotting the same amount of proteins in sample buffer were loaded in each lane. In each experiment, equal proteolipidation was confirmed by Coomasie staining of the nontransfected portion of the gel or membrane staining with Ponceau S. In the immunoprecipitation experiments equal amounts (0.5–1 μg) of protein from different samples were precleared using protein A-Sepharose beads (Amersham Biosciences) for 30 min at 4 °C. One μg of the appropriate antibody was added to the extract and rotated overnight at 4 °C. The immunocomplexes were precipitated with 60–100 μg of protein A-Sepharose beads for 2 h. After centrifugation the pellet was washed twice in lysis buffer and resuspended in sample buffer. Samples were heated at 95 °C for 10 min and the amounts corresponding to ¼ to ½ of the original protein were loaded into 4–12% SDS-polyacrylamide gels, electrophoresed, and transferred to nitrocellulose membranes. The membranes were then probed with the appropriate antibodies. Blots were stripped using a Stripping Kit (Alpha Diagnostic, San Antonio, TX) according to the manufacturer’s protocol.

RSK1 Kinase Assay—For immunocomplex kinase assays, FLAG antibody was added to the nuclear fraction and incubated with rocking for 2 h at 4 °C to the pull down FlagRSK1 protein. The immunocomplexes were precipitated with protein A-Sepharose beads, resuspended in an assay dilution buffer (ADB: 20 mM MOPS, pH 7.2, 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate, and 1 mM dithiothreitol), and the kinase activity was analyzed using an S6 kinase assay kit (Upstate Biotechnology, Inc., Lake Placid, NY). The reaction mixture, containing 50 μM substrate peptide, inhibitor mixture (4 μM protein kinase C inhibitor peptide, 0.4 μM protein kinase A inhibitor peptide, and 4 μM Compound 24571 CDK inhibitor in ADB), and ATP, was incubated for 10 min at 30 °C. Twenty μl of the reaction mixture was spotted onto 1.5 × 1.5 cm sPhos phosphocellulose squares, the papers were washed and radioactivity was measured using a Wallac 1409 scintillation counter. Specific RSK1 activity was calculated by subtracting activity in the absence of the substrate peptide from the total activity.

Immunocytochemistry—Cells were fixed and stained using a FGFR1 polyclonal C-terminal Ab that specifically recognizes the single glycosylated form of endogenous or transfected FGFR1 (11–13, 18, 20, 36, 37, 41) or with monoclonal N-terminal FGFR1 McAb6 (41) that recognizes different glycosylated forms of FGFR1, with sheep polyclonal anti-phospho-Ser364 (rat numbering) RSK1 Ab or with rabbit polyclonal Ab against the C-terminal RSK1 region (Upstate Biotechnology, Lake Placid, NY), as described previously (42).

Fluorescent staining was performed using: 1) Alexa 488-conjugated goat anti-mouse IgG; 2) Alexa Fluor 568-conjugated goat anti-rabbit IgG; 3) Cy3-conjugated donkey anti-mouse; 4) Cy3-conjugated donkey anti-rabbit; or 5) Cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). 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 as previously described (12).

Cells were analyzed using a fluorescent Nikon Diaphot microscope or confocal microscope (13). Confocal sections were acquired with a Bio-Rad MRC 1024 confocal microscope. Colocalization analysis of double labeled cells was performed using Lasersharp version 3.0 from Bio-Rad. The possibility of bleed-through in double fluorescent cells was excluded by background images in sequential mode.

Luciferase Assays—CREB activation was analyzed using pFR-Luc, in which luciferase expression is driven by the GAL4-binding element and pFA2-CREB expressing chimeric GAL4CREB or control PFA2 expressing unfused GAL4 DNA BD (Promega). Cells were transfected with 1 μg of pFR-Luc or PFA2 and 1 μg of each additional plasmid. 48 h after transfection, cells were lysed and analyzed for luciferase activity (20).

Luciferase activity was expressed per microgram of cellular protein and compared with the activity of pCIS-Ck (lacking GAL4-binding element) expressed in parallel plates (42).

Our previous study (21) showed that GAL4CREB increases the expression of pFR-Luc ~7.5-fold compared with the unfused GAL4 BD. Specific stimulation of GAL4CREB-dependent transcription by Flag-RSK1 (Fig. 7B) was determined by calculating the ratio of luciferase activity in cells transfected with FlagRSK1 compared with luciferase activity in cells transfected with the control FLAG plasmid. Potential effects of RSK1 on CREB-independent transcription were taken into account using the following formula: (x/y)z, where indicate luciferase activity with control or LPS treatment.

2 Also, the specific C-terminal or McAb6 FGFR1 immunostaining was confirmed in transfection experiments using TE671 and other cells. Cells transfected with the FGFR1 but not with control pcDNA3.1 vector (15, 20) displayed nuclear FGFR1 immunoreactivity (also see Fig. 5). Immunostaining accumulation of nontransfected FGFR1 was also demonstrated after transfection of FGFR1-myc or FGFR1-EGFP using antibodies against the attached epitope and native EGF fluorescence in live cells (15, 20, 21).
RSK1 and CR1 in yeast is shown in Fig. 1A. RSK1 (aa 36–822), encompassing the Ig Loops, acidic box, and transmembrane domain, and the C-terminal portion (CR1, aa 399–822), encompassing the Ig Loops, acidic box, and transmembrane domain, were used as baits for screening the human fetal brain cDNA library. The MATCH-MAKER 3 two-hybrid was used in which proteins are triple-matched with HIS3, ADE2, and MEL1 promoter-reporter systems to minimize the occurrence of false positives. A total of 3 x 10^6 library clones were screened using CR1 as bait revealing 16 positive cDNA clones, of which were products of two homologous genes: reticulon 1 (RTN1) also known as neuroendocrine-specific protein C or reticulon 1 (RTN3) (Table I). The interaction of FGFR1 with RSK1 was further analyzed.

\[ \text{NSP/RTN1 neuroendocrine-specific protein C or reticulon 1} \]

**Table I**

| Gene isolated      | Bait used | Numbers of clones | Gene fragment length | GenBank number |
|--------------------|-----------|--------------------|----------------------|----------------|
| RSK1               | CR1       | 1                  | 36–735               | 292456         |
| SLAP, Src-like adapter protein | CR1       | 3                  | 1–276                | 1809245        |
| p85α PI3K          | CR1       | 1                  | 19–276               |                |
|                    |           | 1                  | 48–276               |                |
| p85β PI3K          | CR1       | 4                  | 375–724              | 27498202       |
|                    |           | 2                  | 446–724              |                |
| PLC-γ              | CR1       | 1                  | 347–728              | 4826907        |
| SH3BP2             | CR1       | 1                  | 203–1291             | 33598947       |
| NCK2               | CR1       | 1                  | 48–188               | 12730500       |
| KIAA1075           | CR1       | 1                  | 134–381              | 4505347        |
| RTN3, reticulon    | NR1       | 2                  | 31–208               | 307310         |
|                    |           | 4                  | 54–208               |                |
|                    |           | 1                  | 80–208               |                |
|                    |           | 1                  | 87–208               |                |
|                    |           | 1                  | 93–236               | 5174654        |
|                    |           | 2                  | 99–236               |                |
|                    |           | 1                  | 105–236              |                |
|                    |           | 1                  | 118–236              |                |

**RESULTS**

**N- and C-terminal Regions of FGFR1 Interact with Different Proteins**—The N-terminal portion of FGFR1 (NR1, aa 1–401), encompassing the Ig Loops, acidic box, and transmembrane domain, and the C-terminal portion (CR1, aa 399–822), encompassing the split tyrosine kinase domain, were used as baits for screening the human fetal brain cDNA library. The MATCH-MAKER 3 two-hybrid was used in which proteins are triple-screened with HIS3, ADE2, and MEL1 promoter-reporter systems to minimize the occurrence of false positives. A total of 3 x 10^6 library clones were screened using CR1 as bait revealing 16 positive colonies, eight of which represent different genes (Table I). One clone contained the near full-length cDNA of p90 RSK1 (aa 36–735). An example of the interaction between RSK1 and CR1 in yeast is shown in Fig. 1A. Other CR1 interactions included the p85α PI3K, p85β PI3K, phospholipase γ, SH3BP2, the Src-like adapter protein, NCK adaptor protein 2, and KIAA1075 (Table I). One million cDNA library clones were screened using NR1 as bait, revealing 13 positive cDNA clones, which were products of two homologous genes: reticulon 1 (RTN1) also known as neuroendocrine-specific protein and reticulon 3 (RTN3) (Table I). The interaction of FGFR1 with RSK1 was further analyzed.

**NH2-terminal Region of RSK1 Binds to the Cytoplasmic Domain of FGFR1 in Yeast**—To determine the RSK1 domains responsible for its binding to CR1, a series of C- and N-terminal deletion mutants were generated (Fig. 1B, middle panel, marked with 2 to 11). These mutants were cotransformed with pGBT9-CR1 into strain AH109. The RSK1 mutant-CR1 interactions were evaluated with three promoter-reporter systems by screening the survival cells on SD/Ade/His/Leu/Trp/X-gal Medium (shown on Fig. 1B, bottom panel). The four C terminus deletions, KC1 (aa 36–650), KC2 (aa 36–556), KC3 (aa 36–450), and KC4 (aa 36–350), did not affect RSK1 binding to CR1. However, deletion of an additional 25 aa (KC5, aa 36–325) abolished the interaction between RSK1 and CR1. To further map the CR1 binding region we had introduced N-terminal deletions into KC4 (aa 36–350). Deletions of the N-terminal region of RSK1 from amino acids 36 to 71 (KN1 (aa 43–350), KN2 (aa 63–350), KN3 (aa 71–350), did not affect the interaction with CR1. Subsequent deletion of aa 71 to 218 (KN4, aa 218–350) abolished CR1 binding. We also analyzed the activity of α-galactosidase secreted into the medium by the transformed yeast cells. In cultures expressing interacting pairs of proteins, the α-galactosidase activity ranged from 4.2 to 16.8 milliunits per ml of cells. Among them, KN1 (aa 43–350) and CR1 showed the strongest and the KN2 and CR1 had the weakest binding. In cultures expressing the noninteracting pairs of proteins (determined by the survival of the blue colonies in SD/Ade/His/Leu/Trp/X-gal medium), the α-galactosidase activity was equal or lower than 0.7 milliunits/ml. Based on these results we conclude that the RSK1 region essential for FGFR1 binding should start between aa 71 and 218 and extend to the region between aa 335 and 350. This region includes, at least, a part of the NTK and a portion of the interkinase linker region.

**Interaction of FGFR1 with RSK1 in Mammalian Cells and Subcellular Localization of FGFR1-RSK1 Complexes**—The expression of endogenous RSK1 was examined by Western blotting in the nuclear and cytoplasmic fractions of human TE671 medulloblastoma cells transfected with pcDNA3.1-FGFR1 or control pcDNA3.1 (Fig. 2A). TE671 cells express little endogenous FGFR1 and transfected wild type FGFR1 was shown to accumulate both in the nucleus and cytoplasmic fraction (13,
Interaction of FGF Receptor with Ribosomal S6 Kinase-1

**A**

![Diagram showing RSK1 deletions](image)

**B**

![Subcellular localization of endogenous and transfected RSK1 in TE671 medulloblastoma cells](image)

**Fig. 1. Interaction of RSK1 and FGFR1 in yeast.** A, individual BD plasmids (pGBT-9-CR1, pGBK-T7, pGBK-T-7-laminin, or pGBK-T-7-p53) were transformed in yeast strain AH109 and the AD plasmids (pACT2-RSK1, pGAD-T7, and pGAD-T7-SV40 T antigen) were transformed into yeast strain Y187. Transformed AH109 and Y187 strains were mated and selected with SD-Leu/Trp medium. Growth colonies were streaked on the SD-Adh/His-Leu-Trp/X-gal plate. pGADT7-SV40 large T antigen + pGBT-T-p53 were used as a positive control, pGAD7-T7-SV40 large T antigen + pGBT-T-laminin as negative control. Interaction between RSK1 and FGFR1 or p53 and SV40 activated three (HIS3, ADE2, and MEL1) reporter genes and allowed survival of blue colonies. The absence of surviving colonies indicates the lack of interaction between RSK1 and laminin, RSK1 and BD, and laminin and SV40 (negative control). B, identification of the interacting regions. Top panel shows known RSK1 domains: N-terminal kinase domain (NTK), aa 62–321; C-terminal kinase domain (CTK), aa 417–675; two tyrosine phosphorylation sites (T359 and T573), and five serine phosphorylation sites (S154, S221, S363, S380, and S732) are marked. Middle panel shows RSK1 deletions (marked with 2 to 11) and their interaction with CR1. Individual RSK1 plasmids were cotransformed with bait vector pGBT-BD-CR1 into strain AH109. The transformants containing both CR1 and RSK1 mutant plasmids were initially grown in SD-Trp-Leu medium and then were streaked on SD-Adh/His-Leu-Trp/X-gal medium to select for HIS3, ADE2, and MEL1 expression. The results of growth are shown on the bottom panel and also marked with +" and "-" in the middle panel to indicate the presence and absence of the interaction, respectively. The interacting protein pairs were cultured in SD-Leu-Trp medium and the noninteracting pairs in SD-Leu-Trp medium. Activity of secreted α-galactosidase was measured as described under “Experimental Procedures.”

**Fig. 2. Subcellular localization of endogenous and transfected RSK1 in TE671 medulloblastoma cells.** A, cells were transfected with pcDNA3.1 control vector or FGFR1 expressing plasmid. Cytoplasmic (C) and nuclear (N) fractions were isolated 30 h later and immunoblotted with polyclonal C-terminal RSK1 Ab. B, cells were transfected with the indicated pairs of plasmids. Activity of secreted α-galactosidase was measured as described under “Experimental Procedures.”
The cellular fractions were immunoprecipitated with FGFR1 Ab and probed for RSK1 using an anti-FLAG Ab. The association of RSK1 with FGFR1 was observed predominantly in the nucleus and only in cells transfected with both FGFR1 and FlagRSK1 plasmids. Nuclear FlagRSK1 co-immunoprecipitated with the FGFR1 Ab migrated as 88- and 95-kDa bands, which were not observed in samples in which the FGFR1 Ab was replaced with affinity purified control Ab or with Sepharose A beads alone (Fig. 3C). Interestingly, the immunoprecipitation of nuclear proteins with an Ab that targets the C-terminal region of RSK1 pulled down the faster migrating 88 kDa form more effectively than the hyperphosphorylated RSK1 95-kDa form. Such a result could be explained by an obstruction of the C-terminal RSK1 region by ERK in phosphorylated RSK1 (46).

RSK1-FGFR1 binding was also shown in a reverse experiment in which nuclear extracts were immunoprecipitated with the RSK1 Ab and analyzed with the FGFR1 Ab (Fig. 3D). The RSK1 Ab co-immunoprecipitated predominantly hyperglycosylated 130-kDa FGFR1 and to a smaller extent the hypoglycosylated 95- and 105-kDa FGFR1 isoforms in cells transfected with FGFR1. Transfected FGFR1(SP-/NLS), in which the signal peptide has been replaced with the SV40 large T NLS,3 was also co-immunoprecipitated by RSK1 (Fig. 3D). Consistent with FGFR1(SP-/NLS) processing outside the Golgi-ER, this protein was represented by a single nonglycosylated 95-kDa form. In contrast, FGFR1(TK+/H11002) (glycosylated forms of 75 and 85 kDa; Fig. 3E) with the deleted tyrosine kinase domain and C terminus was not co-immunoprecipitated with RSK1 even though its expression levels were higher than FGFR1 as observed also in earlier studies (13, 20). This finding is in agreement with the yeast two-hybrid assay in which RSK1 was detected with the FGFR1 tyrosine kinase bait. To determine whether FGFR1 kinase activity is required for the interaction with RSK1 we generated a FGFR1(K514A) mutant in which the ATP-binding lysine 514 was replaced with alanine. This mutation abolished FGFR1 kinase activity (47) and prevented receptor autophosphorylation.

3 FGFR1(SP-/NLS) is a nonmembrane receptor present exclusively in the cell nucleus. FGFR1(SP-/NLS) stimulates CREB and CBP activities, transcription of different genes, and cellular growth (13, 20, 21).
phorylation.\(^4\) FGFR1(K514A) was expressed at similar levels as the WT FGFR1 and bound to RSK1 (Fig. 3E). Neither FGFR1, FGFR1(SP-/NLS) (Fig. 3D), nor FGFR1(K514A) (not shown) were coimmunoprecipitated with control Ab.

The overall levels of FlagRSK1 expressed in cells co-transfected with wild type FGFR1, FGFR1(K514A), or FGFR1(TK-) were similar. In FGFR1-transfected cells, nuclear FlagRSK1 appears to contain more of the slower migrating protein, suggesting increased RSK1 phosphorylation (see also Fig. 2B).

Interaction of FGFR1 with RSK1 in the Developing Rat Brain—To determine whether the FGFR1-RSK1 interaction also occurs in vivo and between endogenous proteins, an analysis was performed using nuclear and cytoplasmic fractions from the brains of prenatal (E23) rats, a period when intense neuronal growth and differentiation takes place. At E23, FGFR1 localizes both to the cell nuclei and cytoplasm in the rat brain (Fig. 4; also see Ref. 21) and its FGF-2 ligand is exclusively nuclear (21, 48). The RSK1 levels in the prenatal brain were similar in the cytoplasmic and nuclear fractions (Fig. 4A). FGFR1 Ab co-immunoprecipitated more RSK1 in the nuclear fraction than the cytoplasmic fraction, indicating that at this developmental stage RSK1 binds to FGFR1 predominantly in the cell nucleus. The specificity of this binding was confirmed by the lack of FGFR1-RSK1 co-immunoprecipitation with control Ab. In a reverse way, the FGFR1-RSK1 interaction in the cell nuclei of the developing brain was confirmed using the RSK1 Ab for immunoprecipitation and the FGFR1 Ab, McAb6, for Western immunoblotting. RSK1 bound to the most abundant FGFR1 forms (125–130 kDa) (Fig. 4B).

Colocalization of FGFR1 and RSK1—Next we examined whether FGFR1 colocalizes with RSK1 in nuclear and cytoplasmic compartments. TE671 cultures transfected with FGFR1 or pcDNA3.1 were subjected to double immunocytochemical staining for FGFR1 and RSK1 and were analyzed by confocal microscopy. To specifically detect activated (phosphorylated) RSK1, an antibody specific to phospho-Ser\(^{322}\) (40) was used (Fig. 5). Wild type FGFR1 accumulated largely within the nucleus and also within the plasma membrane and submembrane regions (Fig. 5). This distribution was identical to that of native FGFR1-EGFP fluorescence in TE671 (10, 15) and endogenous FGFR1 in cells that express high levels of receptor, which were found in the nuclear interior, plasma membrane, and cytoplasmic vesicles (11–14, 20, 21; see also Fig. 6). Colocalization of FGFR1 and phospho-RSK1 has been observed within the nuclear interior and to a limited extent in the plasma membrane region (Fig. 5). Colocalization of nuclear and cytoplasmic phospho-RSK1 with FGFR1 suggested that the subcellular compartmental distribution of the interaction may be dictated by the presence of FGFR1. Therefore, FGFR1 mutations that restrict its expression to specific compartments were tested to determine whether the pattern of FGFR1-RSK1 colocalization varied according to the compartmental expression of FGFR1. Previous studies showed that the atypical transmembrane domain of FGFR1 is crucial for the release of

\(^4\) Y. Hu, X. Fang, S. M. Dunham, C. Prada, E. K. Stachowiak, and M. K. Stachowiak, unpublished observation.
FGFR1 Stimulates Nuclear RSK1 Activity and RSK1-dependent Activation of CREB—Given the predominant interaction of FGFR1 with RSK1 in the nucleus we examined whether FGFR1 may influence nuclear RSK1 activity. TE671 cells were transfected with the FlagRSK1 expressing plasmid with wild type FGFR1, kinaseinactive FGFR1(K514A), or control pCDNA vector. Thirty hours later, nuclear anti-FLAG immunocomplexes were isolated as shown on Fig. 3E and RSK1 activity was measured (Fig. 7A). RSK1 activity increased ~2.5-fold only in the presence of co-transfected wild type FGFR1, whereas FGFR1(K514A) had no effect. The FGFR1-induced increase in RSK1 kinase activity represents an activation of existing Flag-RSK because the overall levels of FlagRSK1 protein were not affected by cotransfection of FGFR1 (Figs. 2B and 3E).

RSK1 has broad nuclear functions that include phosphorylation and activation of CREB and other transcription factors. The ability of overexpressed nuclear FGFR1 to stimulate CREB Ser133 phosphorylation was shown in our previous study (21). Here we examined whether FGFR1 can regulate CREB activity through RSK1 by transfecting TE671 with RSK1 and with low amounts of FGFR1 that by themselves did not stimulate the CREB activity. CREB activation was analyzed using pFR-Luc in which luciferase expression is directed by the GAL4-binding element and pFA2-CREB expressing chimeric GAL4CREB or control PFA2 expressing unfused GAL4 DNA BD. Our previous study showed that GAL4CREB increases the expression of pFR-Luc ~7-fold, compared with unfused GAL4 BD (21). The individual effects of RSK1, FGFR1, FGFR1(K514A), or FGFR1(TK−) on GAL4CREB-dependent transcription were determined calculating the ratio of luciferase activity in cells transfected with these effector plasmids to the luciferase activity in cells transfected with control vectors (pCMVFlag for FlagRSK1, and pcdNA3.1 for FGFR1, FGFR1(K514A), or FGFR1(TK−) plasmids). The potential effects of RSK1 or receptor-expressing plasmids on CREB-independent transcription were taken into account by using the formulas described under “Experimental Procedures.” The specific GAL4CREB-mediated transcription was increased 2.5-fold by cotransfected FlagRSK1 (Fig. 7B) (RSK1 had no effect on luciferase transcription in the presence of unfused GAL4 BD (not shown)). The GAL4CREB transcriptional activities were not significantly different in cells cotransfected with FGFR1, FGFR1(K514A), or FGFR1(TK−). They were slightly, but not significantly reduced relative to pcDNA3.1 co-transfected cells. Fig. 7C shows the effects of FGFR1 mutants on RSK1 stimulation of CREB-dependent transcription. An additional 2.5-fold increase in FlagRSK1 stimulation of GAL4CREB-mediated transcription was induced by cotransfected FGFR1. In contrast, neither FGFR1(K514A) nor FGFR1(TK−) significantly affected RSK1 stimulation of CREB-mediated transcription. Thus, active FGFR1 and RSK1 stimulate CREB activity in a cooperative manner, an effect that requires active FGFR1 kinase.

**DISCUSSION**

In the present study we have demonstrated an interaction between FGFR1 and RSK1 and their cooperative activity in mammalian cells. The RSK1 domain is essential for binding to FGFR1 overlaps with a part of the NTD and part of the interleukin linker region of RSK1. It includes the Ser133 phosphorylation.
RSK1 has an N-terminal activation loop that is phosphorylated by 3-phosphoinositide-dependent protein kinase 1. The precise N-terminal boundary of the RSK1 region essential for FGFR1 binding and whether it includes the Ser154 phosphorylation site necessary for the binding RSK1 inhibitor 14-3-3/H9252 remain to be determined. The portion of the FGFR1 targeted by RSK1 lies within the region that contains a split TK domain.

The association between FGFR1 and RSK1 was demonstrated in vitro in mammalian medulloblastoma cells and in vivo in the rat brain. We have validated the specificity of the co-immunoprecipitation assays and thus the occurrence of the FGFR1-RSK1 interaction using different combinations of the recombinant or endogenous FGFR1 and epitope-tagged or endogenous RSK1.

In the rat brain, the specific contents of endogenous RSK1 (relative to the total protein content) were similar in the cytoplasmic and nuclear fractions. Also, in TE671 cells, transfected FLAG-RSK1 reached slightly higher levels in the cytoplasm than in the nucleus. Nevertheless, the interaction between FGFR1 and RSK1 in the developing rat brain and between FGFR1 and FlagRSK1 in TE671 cells was observed mainly within the cell nucleus, and to a lesser extent in the cytoplasm. In the TE671 cells this was consistent with the predominantly nuclear distribution of FGFR1 observed in the present study and in earlier studies (10). Our experiments utilizing FGFR1 mutants targeted to specific subcellular compartments have further established that the presence of FGFR1 is the determining factor for the subcellular areas where the interaction with RSK1 may occur. Still, some additional factors may promote the FGFR1-RSK1 interaction in the nucleus, as observed in the developing rat brain in which the RSK1-FGFR1 complex formed predominantly in the nucleus even though the abundance of FGFR1 and RSK1 was similar in the cytoplasm and nucleus.

RSK activation is dependent on the combination of phosphorylation and localization events. RSK1 requires at least two input signals to generate a fully active kinase. First ERK docks at the C terminus, phosphorylates RSK1, and transports the phosphorylated protein to the membrane. Second is the phosphorylation of the N-terminal activation loop by 3-phosphoinositide-dependent protein kinase 1 at the membrane (46). Recent studies have shown that the transient association of RSK1 with the plasma membrane is an important component of generating a fully active RSK1 protein and its subsequent transport to the nucleus (46). The mechanisms that control RSK1 activity within the nucleus are unknown. However, the robust interactions of RSK1 with FGFR1 within the nucleus (Figs. 3–6) and stimulation of nuclear RSK1 (Fig. 7) revealed in the present study imply that a direct encounter of RSK1 with nuclear FGFR1 may control RSK1 activity and RSK1-mediated transcripational regulation.
The predominant association and colocalization of nuclear FGFR1 with more of the phosphorylated form of RSK1, together with the increase in RSK1 phosphorylation caused by FGFR1, suggested that the RSK1 molecules complexed with FGFR1 preferentially undergo phosphorylation. Indeed we found that FGFR1 can induce RSK1 phosphorylation and increase nuclear RSK1 kinase activity, the processes known to be causally related. The present study showed that whereas RSK1 binds to the TK/C-terminal domain, this binding does not require FGFR1 kinase activity. However, the active nuclear FGFR1 tyrosine kinase domain is essential for the activation of RSK1-dependent functions. Our previous studies showed that FGFR1 transfected into TE671 cells accumulates in the nucleus along with the endogenous high molecular weight forms of FGF-2 and undergoes phosphorylation. Hence, nuclear FGFR1 activation by its ligand could account for the kinase activity-dependent stimulation of RSK1 by the transfected wild type receptor.

RSK1 is phosphorylated directly by serine/threonine kinase ERK, but not by tyrosine kinases (23). We detected no tyrosine phosphorylation of RSK1 co-immunoprecipitated with the FGFR1 Ab, whereas cotransfected FGFR1 was tyrosine-phosphorylated (not shown). These results confirmed that FGFR1 does not phosphorylate directly RSK1. It is possible that FGFR1 serves as a scaffold that assembles an ERK-activating complex in which the receptor could activate the Raf/Ras/Mek/ERK cascade (via Ras binding adaptor proteins) and deliver this signal to FGFR1-bound RSK1. Such an action is possible not only in the cytoplasm, but also in the nucleus because all cascade components have been found to be present in the nuclei of stimulated cells (44, 51, 52). Consistent with this model, we found that exclusively transfected nuclear FGFR1(SP-/NLS) increases ERK1/2 phosphorylation in the nuclei of TE671 cells.4

Another potential role of FGFR1 in RSK1 activation may be determined by an interaction between FGFR1 and p85 PI3K (indicated by our yeast two-hybrid analysis). p85 PI3K binding to FGFR1 could lead to local activation of phosphoinositol-dependent 3-phosphoinositide-dependent protein kinase 1, which further phosphorylates and activates RSK1. Also, binding of FGFR1 to the NTK of RSK1 could affect the interaction of RSK1 with 14-3-3β, which negatively regulates RSK1 kinase activity and sequesters RSK1 in the cytosol (50).

One of the known nuclear substrates of RSK is CREB (23). Previously we showed that nuclear FGFR1 can up-regulate the phosphorylated form of CREB (21). In the present study we have demonstrated that RSK1 and nuclear FGFR1 can stimulate CREB in a cooperative (synergistic) manner. Using a GAL4CREB chimera we showed that RSK1 stimulates CREB activity and that RSK1 activation of CREB is markedly enhanced by nuclear FGFR1. Given that CREB is not directly phosphorylated by tyrosine kinases, but is phosphorylated and activated by RSK1 (stimulation specifically by RSK1 was shown in this study), and that nuclear FGFR1 can stimulate RSK1 activity, nuclear FGFR1 is likely to act as an upstream RSK1 activator in the RSK1-activating cascade. In the “feed forward and gate” model of cell activation (10), where diverse intracellular signaling pathways relay transmembrane signals to sequence-specific transcriptional factors, the role of nuclear FGFR1 is to feed forward those signals to the common cotranscriptional activator, CBP/p300, as shown previously (10, 21). The present findings expand the role of nuclear FGFR1 to include activation of RSK1, which in turn activates CREB. Nuclear RSK phosphorylates and activates a number of additional transcriptional factors including ER81 (53), c-Fos (27, 28), C/EBPβ (29), estrogen receptor (31), and IκBα (30, 54). Transcriptional factors that, similar to CREB, are regulated by nuclear FGFR1 include AP1 and NFκB.4 Their activation by nuclear FGFR1 could also involve RSK1.

A potential important consequence of the FGFR1-RSK1 interaction could be activation of CBP. Inactive RSK1 sequesters CBP in an inactive complex and upon RSK1 activation the complex dissociates allowing both RSK1 and CBP to perform their transcriptional functions (55, 56). Thus, RSK1 activation by nuclear FGFR1 could indirectly allow CBP activation. In support of this hypothesis, nuclear FGFR1 was found to be a powerful stimulator of CBP-mediated transcriptional activity (21).

Given the diversity of RSK substrates and their cellular roles, there has been great interest in understanding the regulation of RSK activity. Our studies imply that FGFR1 may control some of the functions of RSK1 via a direct interaction with the protein. This interaction would provide a new level of control over RSK1 effects and provide a mechanism by which nuclear FGFR1 exerts its effects on cellular development and homeostasis. Further studies are necessary to elucidate how the FGFR1-RSK1 interaction affects fundamental cellular processes initiated inside and outside of the nucleus.

In addition to RSK1, nine proteins have been identified as capable of binding to the N or C terminus of FGFR1 in yeast cells. The interaction between p85 PI3K and FGFR, or NCK and FGFR1 has only been shown in Xenopus (7). Our results show that this interaction may occur between mammalian proteins as well. p85 PI3K similar to RSK1 is found both in the cytoplasm and the nucleus (23, 46, 57) and thus could serve as the binding partner for both the nuclear and extranuclear FGFR1. FGFR1 binding to the phospholipase γ SH2 domain has been shown previously (3, 4). Adaptor NCK2 (58), p85 PI3K (α and β) (59, 60), Src-like adapter protein (61, 62), SH3 binding domain protein (SH3BP2) (63), and KIAA1075 (64) all contain SH2 domains that potentially could interact with the FGFR1 TK and adjacent photophosphotyrosines. RTN1 and RTN3, which interact with the N-terminal portion of FGFR1, associate with the ER membranes (65–67). RTN-1 aggregates form a channel-like structure, and thus could potentially play a role in protein movement in and out of the membrane as found with FGFR1 (15). The results of our yeast two-hybrid screening revealed a complexity of the potential FGFR1 interactions with the cellular proteins that extend beyond the classical tyrosine kinase receptor signaling. Whereas the FGFR1 interactions shown by the triple promoter-reporter two-hybrid assays are likely to be specific, their occurrence and functional significance in mammalian cells remains to be established as we have done for FGFR1 interaction with RSK1.

Acknowledgments—We thank Dr. Pamela A. Maher (Scripps Research Institute, La Jolla, CA) for pGBK7 FGFR1 (CR1) plasmid and for C-terminal FGFR1 Ab, Dr. Dennis Higgins (Department of Pharmacology, SUNY, Buffalo, NY) for providing E23 rat embryos, and Drs. Leonora Buzanska and Krystyna Domanska-Janik (Polish Academy of Sciences, Warsaw, Poland) for HUCB-NSC cells.

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Yafang Hu, Xiaohong Fang, Star M. Dunham, Claudia Prada, Ewa K. Stachowiak and Michal K. Stachowiak

J. Biol. Chem. 2004, 279:29325-29335.
doi: 10.1074/jbc.M311144200 originally published online April 26, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M311144200

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