Association of Fibroblast Growth Factor Receptor 1 with the Adaptor Protein Grb14

CHARACTERIZATION OF A NEW RECEPTOR BINDING PARTNER*

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Using the cytoplasmic domain of fibroblast growth factor receptor 1 (FGFR1) as bait in a yeast two-hybrid screen, Grb14 was identified as a FGFR1 binding partner. A kinase-inactive mutant of FGFR1 failed to interact with Grb14, indicating that activation of FGFR1 is necessary for binding. Deletion of the C-tail or mutation of both C-tail tyrosine residues of FGFR1 to phenylalanine abolished binding, and deletion of the juxtamembrane domain of the receptor reduced binding, suggesting that Grb14 binds to FGFR1 at multiple sites. Co-immunoprecipitation and in vitro binding assays demonstrated that binding of Grb14 to FGFR1 in mammalian cells was dependent on receptor activation by fibroblast growth factor-2 (FGF-2). Deletion of the Src homology 2 (SH2) domain of Grb14 reduced but did not block binding to FGFR1 and eliminated dependence on receptor activation. The SH2 domain alone bound both FGFR1 and platelet-derived growth factor receptor, whereas full-length Grb14 bound only FGFR1, suggesting that regions upstream of the SH2 domain confer specificity for FGFR1. Grb14 was phosphorylated on serine and threonine residues in unstimulated cells, and treatment with FGF-2 enhanced this phosphorylation. Expression of exogenous Grb14 inhibited FGF-2-induced cell proliferation, whereas a point-mutated form of Grb14 incapable of binding to FGFR1 enhanced FGF-2-induced mitogenesis. These data demonstrate an interaction between activated FGFR1 and Grb14 and suggest a role for Grb14 in FGF signaling.

The fibroblast growth factors (FGFs) constitute a rapidly expanding family of polypeptides with mitogenic and morphogenic effects on a wide variety of cell types (1). Basic FGF (FGF-2) represents one of the prototype members of this family. The FGFs function through a dual-receptor system consisting of high affinity tyrosine kinase receptors (FGFR) and low affinity receptors composed of cell surface heparan sulfate proteoglycans (2). Four FGFR genes have been cloned to date, each coding for a transmembrane protein with a cytoplasmic tyrosine kinase domain (3). As with many growth factor receptors, binding of ligand results in receptor dimerization, which leads to activation of the cytoplasmic kinase domain. Following these events, intracellular proteins are recruited to the receptor and carry out the downstream signal transduction events.

A limited number of cytoplasmic proteins that bind to FGFR1 have been identified. These include phospholipase Cγ, FGF receptor substrate 2, and Shc (4–6). Shc and FGF receptor substrate 2 function as adaptor proteins, linking FGF activation to cytoplasmic kinase cascades such as the FGF receptor substrate 2-mediated association with Grb2-Sos followed by the association of Ras and downstream activation of the mitogen-activated protein kinase cascade. Activation of FGFR and the subsequent signal transduction events are involved in the regulation of a wide variety of cellular processes, and it is likely that other cytoplasmic proteins participate in these responses. The present study was undertaken to identify additional components of FGF signal transduction pathways.

The cytoplasmic domain of FGFR1 (CD-R1) was used as bait in a two-hybrid screen of a mouse cDNA library. One of the proteins identified in this screen was the murine homolog of human growth factor receptor-bound protein 14 (Grb14). Grb14 is a member of an emerging family of noncatalytic adaptor proteins that also includes Grb7 and Grb10 (7). Characteristic features of Grb7 family members include a central pleckstrin homology (PH) domain, a C-terminal Src homology 2 (SH2) domain, and an N-terminal proline-rich motif, which conforms to the consensus sequence of a SH3 binding site. The presence of these functional domains indicates that Grb7 family members have the potential to interact with a variety of proteins. Grb7 binds to several members of the ErbB family of receptor tyrosine kinases as well as to platelet-derived growth factor receptor (PDGFR), SH2-containing protein tyrosine phosphatase 2, focal adhesion kinase, and both the Ret and Tek/Tie2 receptor tyrosine kinases (8–14). Grb10 is expressed as several splice variants that have been shown to bind differentially to the insulin receptor (IR), insulin-like growth factor I receptor, PDGFR, epidermal growth factor receptor (EGFR), Ret, EphB1/ELK, growth hormone receptor, Nedd4, Raf1, MEK1 (mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1), and the c-Abl and Tec nonreceptor tyrosine kinases (15–26). Grb14 has been shown to bind to Tek/Tie2, PDGFR, and IR (14, 27, 28). The large number of binding partners for the Grb7 family members suggests that they play key roles as cytoplasmic signaling intermediates. The present study describes a novel interaction between Grb14 and FGFR1.
**EXPERIMENTAL PROCEDURES**

**Yeast Two-Hybrid Screen**—These experiments used the Matchmaker GAL4 yeast two-hybrid system and an NIH/3T3 cDNA library (CLONTECH, Palo Alto, CA). The cDNA coding for the cytoplasmic domain of FGFFR1 (amino acids 399–822) was cloned into pAS2–1 and pGBT9 DNA binding domain vectors (high and low level expression, respectively). High expression levels of CD-R1 were toxic to yeast, so subsequent experiments were carried out with CD-R1 in pGBT9. Since the manufacturer of the two-hybrid system does not recommend use of 3-amino-1,4-triazole with pGBT9, the leaky HIS3 gene expression allowed growth of colonies expressing noninteracting pairs of proteins. Determination of positive interactions was by filter-lift β-galactosidase assay, according to the manufacturer’s protocol. Briefly, filter lifts were snap-frozen in liquid N₂, thawed, and placed in contact with filters presoaked with X-gal. Filters were incubated for 5 days at 30 °C. Positively clones were identified by formation of blue product in less than 6 h. CD-R1 deletion mutants lacking the juxtamembrane domain (amino acids 399–476) or the C-tail (amino acids 756–822) were generated by polymerase chain reaction (PCR), and point mutants were generated by PCR-based site-directed mutagenesis. All constructs were verified by DNA sequencing.

**Cloning of Mouse Grb14**—One of the positive clones from the yeast two-hybrid screen coded for an in-frame, N-terminally truncated murine homolog of human Grb14. To obtain the complete mouse cDNA, 5′-rapid amplification of cDNA ends (RACE) was performed using a mouse liver 5′RACE cDNA library (CLONTECH). MEsO (5%) was added to all reactions to overcome the >90% GC content of the 5′ end of the cDNA. Linear amplification of the library was performed using XhoI primers responding to the same sequence near the 5′ end of the truncated clone, then the 5′ end of the Grb14 cDNA was PCR-amplified with a nested Grb14 primer and a 5′RACE primer. Following sequencing of the 5′-RACE product, the entire Grb14 cDNA was amplified from the same library using the Advantage-Hi Fidelity polymerase mix (CLONTECH), and two independent clones were completely sequenced on both strands. The coding region was subcloned into the pACT2 GAL4 activation domain vector for use in the yeast two-hybrid assay.

**Cell Culture and Preparation of Lysates**—PA1 cells were obtained from ATCC (Manassas, VA) and maintained in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies, Inc.) supplemented with 10% fetal bovine serum (HyClone, Ogden, UT). Before treatment, confluent cells were washed in serum-free DMEM then starved for 48 h in DMEM supplemented with 0.5% serum. Cells were treated with recombinant human FGF-2 (25 ng/ml) or HGF (20 ng/ml) for 3 h. To measure DNA synthesis, cells were labeled for 3 h with 3H-thymidine (ICN) and subsequent incubation for 5 h. Cells were washed twice in phosphate-buffered saline and scraped into lysis buffer. Lysates were cleared by centrifugation, and Grb14 was immunoprecipitated using a monoclonal antibody against FGFR (mAb6) and subjected to partial acid hydrolysis according to the method of Kamps and Sefton (29). Phosphoamino acid analysis and immunoprecipitation of Grb14 was transferred to polyvinylidene difluoride (Millipore, Bedford, MA) and subjected to partial acid hydrolysis according to the above described methods. Nuclear run-on transcription was performed as described above.

**Preparation of Stable Cell Lines and Measurement of DNA Synthesis**—For establishment of stable lines, NIH/3T3 cells were transfected with pcDNA3.1/HisA, pcDNA3.1/HisA/Grb14, or pcDNA3.1/HisA/Grb14 R466K as described above, maintained for 48 h in DMEM-supplemented 10% calf serum, then passed into media containing 800 µg/ml G418 (Life Technologies, Inc.). Cells were grown under selection.

**In Vivo Interaction Studies**—The coding region of Grb14 was amplified from a human liver cDNA library (CLONTECH) by PCR. MEsO (5%) was added to all reactions to overcome the >90% GC content of the 5′ end of the cDNA. Full-length Grb14 was subcloned in-frame into pcDNA3.1/HisA (Invitrogen, Carlsbad, CA) for expression of epitope-tagged Grb14. The R466K point mutation was generated by PCR-based site-directed mutagenesis according to standard protocols.

For transient transfections, NIH/3T3 cells were seeded at 1.8 × 10⁴ cells/cm² and grown for 18 h in DMEM supplemented with 10% calf serum. Cells were transfected with pcDNA3.1/HisA, pcDNA3.1/HisA/Grb14, or pcDNA3.1/HisA/Grb14 R466K in the presence of 10% serum for 24 h using Effectene transfection reagent (Qiagen, Valencia, CA) according to the manufacturer’s protocol. Cells were then washed with serum-free DMEM, starved for 24 h in DMEM supplemented with 0.5% serum, and treated with recombinant human FGF-2 (25 ng/ml) for 10 min at 37 °C. Lysates were prepared, and protein concentrations were determined as described above.

**For Immunoprecipitation of FGFFR1**—200 µg of each cell lysate were incubated with 2 µg of a polyclonal antibody against FGFFR1 (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C, and immune complexes were collected on protein A-Sepharose (Amersharm Pharmacia Biotech). Complexes were washed twice in a buffer containing 20 mM HEPES, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol, then once in phosphate-buffered saline. Bound proteins were eluted by boiling in 2× Laemmli sample buffer, separated by SDS-polyacrylamide gel electrophoresis (PAGE), and transferred to nitrocellulose. Membranes were blotted with a monoclonal antibody against FGFFR (mAb6) generated in this laboratory or a monoclonal antibody against the pcDNA3.1/HisA Xpress epitope tag (Santa Cruz Biotechnology), followed by appropriate secondary antibodies. Immunoreactive bands were visualized with enhanced chemiluminescence (ECL) using SuperSignal substrate (Pierce).

**Generation of hGrb14 Fusion Proteins**—Full-length Grb14 or DNA fragments corresponding to the desired fusion proteins were subcloned in-frame into pGEX-5X-1 (Amersham Pharmacia Biotech) for expression as glutathione S-transferase (GST) fusion proteins. GST fusion proteins were expressed in Escherichia coli strain BL21 and purified using glutathione-Sepharose (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. All constructs were verified by DNA sequencing, and fusion proteins were verified by SDS-PAGE and Western blotting with antibodies against GST (Amersham Pharmacia Biotech) and Grb14 (Transduction Laboratories, Lexington, KY and Santa Cruz Biotechnology).

**In Vitro Binding Assays**—Purified GST fusion proteins or GST alone (5 µg) were immobilized on glutathione-Sepharose (Sigma) over night at 4 °C with 1.0 mg of each cell lysate. Precipitated proteins were extensively washed in phosphate-buffered saline, and bound proteins were eluted by boiling in 2× Laemmli sample buffer, separated by SDS-PAGE, and transferred to nitrocellulose. Membranes were blotted with a monoclonal antibody against FGFFR (mAb6) or a polyclonal antibody against PDGFR (Upstate Biotechnology, Lake Placid, NY), followed by appropriate secondary antibodies. Immunoreactive bands were visualized with ECL using SuperSignal substrate.

**Metabolic Labeling and Phosphoamino Acid Analysis**—Confluent PA1 cells were starved in DMEM supplemented with 0.5% serum for 48 h, then washed twice in phosphate-free DMEM (Life Technologies, Inc.) supplemented with 0.5% dialyzed fetal bovine serum (HyClone). Cells were labeled for 3 h with the same medium containing 100 µCi/ml [3H]orthophosphate (ICN, Costa Mesa, CA), then treated with recombinant human FGF-2 (25 ng/ml) for 10 min at 37 °C. Cells were washed twice in phosphate-buffered saline and scraped into lysis buffer. Lysates were cleared by centrifugation, and Grb14 was immunoprecipitated using a commercial antibody (Santa Cruz Biotechnology) and analyzed by SDS-PAGE and autoradiography. For phosphoamino acid analysis, immunoprecipitated Grb14 was transferred to polyvinylidene difluoride (Millipore, Bedford, MA) and subjected to partial acid hydrolysis according to the method of Kamps and Sefton (29). Phosphoamino acids were separated by two-dimensional electrophoresis on 0.1-mm cellulose thin-layer chromatography plates (EM Science, Gibbstown, NJ) according to published protocols (30) and detected by autoradiography.

**Preparation of Stable Cell Lines and Measurement of DNA Synthesis**—For establishment of stable lines, NIH/3T3 cells were transfected with pcDNA3.1/HisA, pcDNA3.1/HisA/Grb14, or pcDNA3.1/HisA/Grb14 R466K as described above, maintained for 48 h in DMEM-supplemented 10% calf serum, then passed into media containing 800 µg/ml G418 (Life Technologies, Inc.). Cells were grown under selection for 3 weeks, and individual clones were established according to standard protocols, screened for expression of epitope-tagged Grb14, and maintained under selection.

For measurement of DNA synthesis, vector and Grb14 stable lines were grown to confluence in 96-well dishes in DMEM supplemented 10% calf serum and 800 µg/ml G418 (Life Technologies, Inc.). Cells were grown under selection. For establishment of stable lines, NIH/3T3 cells were transfected with pcDNA3.1/HisA, pcDNA3.1/HisA/Grb14, or pcDNA3.1/HisA/Grb14 R466K as described above, maintained for 48 h in DMEM-supplemented 10% calf serum, then passed into media containing 800 µg/ml G418 (Life Technologies, Inc.). Cells were grown under selection for 3 weeks, and individual clones were established according to standard protocols, screened for expression of epitope-tagged Grb14, and maintained under selection.

**RESULTS**

**Identification of Grb14 by Interaction with FGFFR1 in a Yeast Two-hybrid Screen**—An NIH/3T3 cDNA library was screened using the cytoplasmic domain of FGFFR1 (CD-R1) as bait. The DNA sequence of one of the positive clones was 84% identical to a portion of human Grb14 and represents the murine homolog. The full-length mouse Grb14 cDNA was cloned using 5′-RACE,
and the DNA sequence and predicted amino acid sequence are shown in Fig. 1. Mouse Grb14 cDNA encodes a 538-amino acid protein (mGrb14) that is likely to function as an adaptor. Several protein-protein interaction domains are present, including an SH2 domain, a PH domain, and a proline-rich motif representing a putative SH3 binding site. Alignment of the amino acid sequences of Grb14 from mouse, rat (28), and human (27) reveals a 93% sequence identity between mouse and rat and an 85% sequence identity between mouse and human, with the majority of the nonconserved residues in the N-terminal region of the protein (Fig. 2).

Binding of Grb14 Requires FGF Kinase Activity and a C-tail Tyrosine Residue—The interaction between FGFR1 and Grb14 was further characterized using the yeast two-hybrid assay. A series of deletion and point mutation constructs of CD-R1 was tested against mGrb14 (Fig. 3). The CD-R1 construct is a constitutively active tyrosine kinase, as determined by antiphosphotyrosine immunoblotting of a bacterially expressed GST fusion construct (data not shown). A point mutation that inactivates the kinase (K514M) completely abolished binding of CD-R1 to Grb14. This indicates that kinase activity is required for binding of Grb14 and suggests a role for receptor autophosphorylation in the interaction. Deletion of the juxtamembrane region of the receptor (D JM) reduced the strength of the interaction, whereas deletion of the C-tail (D C) abolished binding to Grb14. These deletions had no effect on the kinase activity of the receptor constructs, as determined by antiphosphotyrosine immunoblotting of bacterially expressed GST fusion constructs (data not shown). These data indicate that the C-tail of the receptor is required for binding of Grb14 and that the juxtamembrane region is also involved, suggesting two possible sites of interaction.

The C-tail of FGFR1 contains two tyrosine residues, at positions 766 and 776. Independent mutation of either of these residues to phenylalanine did not affect the ability of CD-R1 to bind to Grb14 in the yeast two-hybrid assay (Fig. 3). However, mutation of both Tyr-766 and Tyr-776 to phenylalanine completely abolished the interaction with Grb14. SH2 domain-containing adaptor proteins typically bind to phosphotyrosine residues, as seen in the binding of phospholipase Cγ to the C-tail of FGFR1 autophosphorylated on Tyr-766 (6). The binding of Grb14 to the independent Y766F and Y776F constructs suggests that Tyr-776 is also autophosphorylated and that Grb14 can recognize and bind to FGFR1 when either Tyr-766 or Tyr-776 is phosphorylated.

FGF-dependent in Vivo Association of FGFR1 and Grb14—To verify that the interaction between FGFR1 and Grb14 identified in yeast also occurs in mammalian cells, NIH/3T3 fibroblasts were transiently transfected with plasmids expressing epitope-tagged hGrb14. Grb14 was co-immunoprecipitated with FGFR1 from lysates of cells treated with FGF-2 but not from untreated cells (Fig. 4). These data confirm an in vivo interaction between Grb14 and FGFR1 that is dependent upon receptor activation. A motif conserved across SH2 domains (FLVRES) is critical for binding to phosphotyrosine. Mutation of the arginine residue within this motif to lysine disrupts binding of SH2 domains to phosphotyrosine (32). This mutation (R466K) prevented the co-immunoprecipitation of Grb14 with FGFR1 from lysates of FGF-treated cells (Fig. 4), suggesting that autophosphorylation of FGFR1 on tyrosine is necessary for the binding of Grb14 in vivo, as shown in the yeast two-hybrid assay. Immunoprecipitation was confirmed by blotting for FGFR1, which was detected as proteins of 155, 145, and 120 kDa, representing the 3 and 2 immunoglobulin-like (Ig) domain forms of the receptor with varying degrees of glycosylation.

Fig. 1. Mouse Grb14 cDNA and predicted amino acid sequence. Nucleotide and predicted amino acid sequence of mouse Grb14. The coding nucleotide sequence is represented in uppercase type, and the 5′- and 3′-untranslated regions are represented in lowercase type. The proline-rich motif conserved among Grb7 family members is indicated by a double underline. The PH domain is underlined, and the SH2 domain is shown in boldface type. The translational termination codon is indicated by an asterisk. Numbers refer to distances in bp.
Grb14 Interacts with FGFR1 via Its SH2 Domain and Its N-terminal Domain—The roles of the different domains of Grb14 in binding to FGFR1 were investigated using in vitro pull-down assays. A series of deletion and point mutation constructs of hGrb14 fused to GST (Fig. 5A) were immobilized on glutathione-Sepharose and used to pull down FGFR1 from lysates of control and FGF-2-treated PA1 cells. In PA1 lysates, FGFR1 was detected as a 155-kDa protein representing the glycosylated 3 Ig domain form of the receptor, with a small amount of the 2 Ig domain form present as a 130-kDa protein. Using lysates of untreated cells (Fig. 5B), only the N-terminal domain (N) and a construct lacking the SH2 domain of Grb14 (D1SH2) were able to bind to FGFR1. In contrast, using lysates of cells treated with FGF-2 (Fig. 5C), full-length Grb14 (WT) bound to FGFR1 strongly, whereas weaker binding was detected with the N-terminal domain (N), constructs lacking either the N-terminal domain (ΔN) or the SH2 domain (ΔSH2), and the SH2 domain alone (SH2). The R466K point mutation abolished binding to FGFR1 of all SH2 domain-containing Grb14 constructs (Fig. 5C), as did an alternate point mutation (F463L) within the FLVRES motif (data not shown). In all cases, no binding of GST to FGFR1 was observed. These data support a model of Grb14 binding to FGFR1 in which the SH2 domain of Grb14 recognizes a tyrosine residue that is autophosphorylated after receptor activation. Regions of Grb14 upstream of the SH2 domain are capable of binding to FGFR1 regardless of the activation state of the receptor, as demonstrated by the binding of both the N-terminal domain and the construct lacking the SH2 domain to FGFR1 from lysates of both untreated and FGF-2-treated cells. However, the presence of the SH2 domain prevented binding of Grb14 to FGFR1 from lysates of untreated cells, and the R466K point mutation in the SH2 domain blocked binding to FGFR1 from lysates of FGF-2-treated cells.

Since the SH2 domain of Grb14 was previously shown to bind to PDGFR and was cloned via interaction with the EGFR C-tail (27), the binding of several domains of Grb14 to FGFR1, PDGFR, and EGFR was compared. These experiments used NIH/3T3 cells since they express higher levels of PDGFR than PA1 cells. No binding to EGFR was detected (data not shown). As in PA1 cells, binding of Grb14 constructs containing an SH2 domain to PDGFR was also investigated (data not shown).
domain to FGFR1 from lysates of untreated cells was not observed (Fig. 6A). The strongest binding to FGFR1 from lysates of FGF-2-treated cells was seen with full-length Grb14. Deletion of the N-terminal domain reduced but did not prevent binding, and the SH2 domain alone also bound weakly to FGFR1. None of the constructs bound to PDGFR from lysates of untreated cells (Fig. 6B). However, the SH2 domain alone bound strongly to PDGFR from lysates of NIH/3T3 cells treated with PDGF. This is in agreement with previously published results (27), which demonstrated a similar interaction between the Grb14 SH2 domain and PDGFR in lysates of PDGF-treated HER14 cells. However, neither full-length Grb14 nor the construct lacking the N-terminal domain bound to PDGFR from lysates of cells treated with PDGF (Fig. 6B). This suggests that the determinants of receptor specificity for Grb14 are localized to regions upstream of the SH2 domain.

**Phosphorylation of Grb14**—Activation of FGFR1 results in direct and indirect phosphorylation of multiple cytoplasmic proteins. Metabolic labeling with \(^{32}P\)orthophosphate was used to assess the phosphorylation state of Grb14 before and after treatment of cells with FGF-2 (Fig. 7A). In unstimulated cells, Grb14 exhibited a basal level of phosphorylation, which was increased 1.8-fold with FGF-2 treatment. Consistent with previously reported data (27), treatment with PDGF resulted in increased Grb14 phosphorylation of a slightly smaller magnitude than that seen with FGF-2, and EGFR treatment did not affect Grb14 phosphorylation (data not shown). Two-dimensional phosphoamino acid analysis demonstrated that Grb14 was phosphorylated primarily on serine residues, with a minor degree of phosphorylation evident on threonine residues (Fig. 7B). Previously published experiments (27) showed PDGF-stimulated Grb14 phosphorylation exclusively on serine. The threonine phosphorylation described in the present study may be specific to FGF-2 stimulation. Alternatively, the present observation may result from increased sensitivity of detection, as evidenced by the shorter autoradiographic exposures necessary for the development of signal. Other members of the Grb7 family are also phosphorylated in both unstimulated and growth factor-treated cells; Grb7 is phosphorylated on serine and threonine (9), and Grb10 is phosphorylated on serine (15). The absence of PDGF-induced tyrosine phosphorylation of Grb14 indicates that it is not a substrate of the FGFR tyrosine kinase and suggests that a downstream component of the FGF signal transduction pathway is required for Grb14 phosphorylation.

**Effects of Exogenous Grb14 Expression on FGF-stimulated DNA Synthesis**—To test the effects of exogenous Grb14 expression on FGF-induced cell proliferation, stable NIH/3T3 cell lines were generated expressing wild-type Grb14 or Grb14 with the R466K point mutation. Individual clones selected for analysis expressed comparable levels of wild-type and mutant Grb14, as determined by immunoblotting for the epitope-tagged exogenous protein (data not shown). Cell lines expressing wild-type Grb14 demonstrated reduced proliferation in response to FGF-2 compared with vector controls, as measured by \(^{3}H\)thymidine incorporation (Fig. 8). This suggests that endogenous Grb14 plays an inhibitory role in FGF signaling. Cell lines expressing Grb14 R466K responded more strongly to FGF-2 than vector controls, showing significantly greater \(^{3}H\)thymidine incorporation, even at low doses of the growth factor. Since Grb14 R466K does not bind to FGFR1, the potentiality of FGF-induced proliferation is likely due to interactions with as yet undetermined downstream effectors. The

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**FIG. 3.** Binding of Grb14 to FGFR1 in the yeast two-hybrid assay. Deletion and point mutation constructs of CD-R1 were tested for interaction with mGrb14 in the yeast two-hybrid assay. The K514M point mutation inactivates the tyrosine kinase of FGFR1 and prevents binding to Grb14. Deletion of the C-tail also prevents binding, as does mutation of both C-tail tyrosine residues to phenylalanine but not mutation of only a single C-tail tyrosine residue. Strength of interaction is scored as moderate (+) or high (+++) based on speed and intensity of color development in a filter lift \(\beta\)-galactosidase assay; – indicates no detectable color development. The following features of FGFR1 are indicated: S, signal sequence; Ig, immunoglobulin-like domains; A, acid box; T, transmembrane domain; JM, juxtamembrane domain; split kinase domain; C, C-tail; and sites of point mutations.

**FIG. 4.** In vivo binding of FGFR1 to Grb14. NIH/3T3 cells were transiently transfected with control vector or with constructs expressing wild-type (WT) or R466K (RK) Grb14 with an Xpress epitope tag. The R466K point mutation disrupts a motif conserved across all SH2 domains that is required for binding to phosphotyrosine (32). FGFR1 was immunoprecipitated (IP) from lysates of untreated (–) or FGF-2-treated (+) cells. After washing, immune complexes were separated by SDS-PAGE, transferred to nitrocellulose, blotted with monoclonal antibodies against the Xpress epitope tag or FGFR, and detected with ECL.
addition of $[^3]$H]thymidine at 12, 18, 24, 36, or 48 h following the onset of FGF treatment revealed no difference in timing of FGF-stimulated DNA synthesis among the cell lines tested (data not shown). All three cell lines demonstrated a similar, strong response to 10% serum (Fig. 8), indicating that the strength equal to the wild-type CD-R1. Previous studies have demonstrated that Tyr-766 is autophosphorylated and that this phosphotyrosine residue is the binding site for the SH2 domain of phospholipase Cγ (6). The Y766F point mutation inhibits the binding and activation of phospholipase Cγ and inhibits receptor internalization (33–35). However, this mutation has no effect on FGF-induced proliferation or differentiation (34–36). One reason that the FGFR1 Y766F mutant retains the ability to transduce FGF-induced signals is the binding of non-SH2 domain-containing adaptor proteins such as FGF receptor substrate 2, which binds to the juxtamembrane domain of the receptor (37). It is also possible that Grb14 and potentially other SH2 domain-containing adaptor proteins can bind to FGFR Y766F and link the activated receptor to intracellular-signaling cascades. Since the present study demonstrates a requirement for receptor activation in the binding of SH2 domain-containing constructs of Grb14, a phosphotyrosine residue in the receptor is likely to be involved, although this may not be an absolute requirement, since the binding of SH2 domains to proteins lacking phosphotyrosine has been reported (38, 39). Early studies on the autophosphorylation of FGFR1 suggested that Tyr-776 is not autophosphorylated (6). However, the methods utilized in this study failed to detect other tyrosine residues that were subsequently shown to be autophosphorylated (40), and these later studies do not rule out the possibility of autophosphorylation on Tyr-776. Furthermore, it is possible that even if Tyr-776 is not autophosphorylated in the wild-type receptor, it may be autophosphorylated in the Y766F mutant. The binding of Grb14 to the Y766F mutant independently of receptor phosphorylation. Furthermore, different domains of Grb14 bind to FGFR1 relatively weakly, whereas full-length Grb14 interacted strongly. This suggests a synergistic binding of different Grb14 domains to FGFR1, as has been observed in the binding of Grb10 to the IR (19).

A mutant CD-R1 with both C-tail tyrosine residues mutated to phenylalanine failed to bind to Grb14; however, the independent Y766F and Y776F receptor constructs bound to Grb14 with strength equal to the wild-type CD-R1. Previous studies have demonstrated that Tyr-766 is autophosphorylated and that this phosphotyrosine residue is the binding site for the SH2 domain of phospholipase Cγ (6). The Y766F point mutation inhibits the binding and activation of phospholipase Cγ and inhibits receptor internalization (33–35). However, this mutation has no effect on FGF-induced proliferation or differentiation (34–36). One reason that the FGFR1 Y766F mutant retains the ability to transduce FGF-induced signals is the binding of non-SH2 domain-containing adaptor proteins such as FGF receptor substrate 2, which binds to the juxtamembrane domain of the receptor (37). It is also possible that Grb14 and potentially other SH2 domain-containing adaptor proteins can bind to FGFR Y766F and link the activated receptor to intracellular-signaling cascades. Since the present study demonstrates a requirement for receptor activation in the binding of SH2 domain-containing constructs of Grb14, a phosphotyrosine residue in the receptor is likely to be involved, although this may not be an absolute requirement, since the binding of SH2 domains to proteins lacking phosphotyrosine has been reported (38, 39). Early studies on the autophosphorylation of FGFR1 suggested that Tyr-776 is not autophosphorylated (6). However, the methods utilized in this study failed to detect other tyrosine residues that were subsequently shown to be autophosphorylated (40), and these later studies do not rule out the possibility of autophosphorylation on Tyr-776. Furthermore, it is possible that even if Tyr-776 is not autophosphorylated in the wild-type receptor, it may be autophosphorylated in the Y766F mutant. The binding of Grb14 to the Y766F mutant

**DISCUSSION**

In this study, we demonstrate the activation-dependent binding of FGFR1 to Grb14 in the yeast two-hybrid assay and using co-immunoprecipitation and *in vitro* pull-down assays. Deletion of the C-tail of FGFR1 abolished binding to Grb14, and deletion of the juxtamembrane region of the receptor reduced the strength of the interaction. Since neither deletion affected the kinase activity of the receptor, it is likely that both regions are involved in the binding of FGFR1 to Grb14. The requirement for receptor activation is consistent with receptor binding of the Grb14 SH2 domain, which specifically recognizes phosphotyrosine residues in the correct sequence context. Mutation of both C-tail tyrosine residues to phenylalanine abolished binding, suggesting that the SH2 domain of Grb14 binds to the C-tail of the receptor. However, deletion of the SH2 domain confers on Grb14 the ability to bind to FGFR1 independently of receptor phosphorylation. Furthermore, different domains of Grb14 bound to FGFR1 relatively weakly, whereas full-length Grb14 interacted strongly. This suggests a synergistic binding of different Grb14 domains to FGFR1, as has been observed in the binding of Grb10 to the IR (19).
obtained in two independent experiments.

with \([3H]\)thymidine and analyzed by liquid scintillation counting of incorporations. Cells were then labeled for 5 h and, Cherenkov radiation was counted. Values are expressed as fold increase over untreated cells and represent the mean ± S.E. of three independent experiments. B, Grb14 bound to polyvinylidene difluoride was subjected to partial amino acid hydrolysis and two-dimensional phosphoamino acid analysis as described under “Experimental Procedures.” Mobilities of phosphoamino acid standards phosphoserine (pSer), phosphothreonine (pThr), and phosphotyrosine (pTyr) are indicated. Exposure time was 3 days at ~80 °C. Similar results were obtained in two independent experiments.

Another common feature of studies on the targets of SH2 domain-containing proteins is the use of fusion proteins of only the SH2 domain. The SH2 domain of Grb14 was initially isolated with a cloning strategy using the activated C-tail of EGFR (27), and in the present study, the SH2 domain of Grb14 was capable of binding both FGFR1 and PDGFR from lysates of growth factor-treated cells. Similarly, the SH2 domains of Grb7 and Grb10 have been shown to bind multiple growth factor receptors and other targets (7). The ability of isolated SH2 domains to bind multiple targets complicates interpretation of the roles of these adaptors. However, the present study demonstrates that the target specificity of full-length Grb14 is more stringent than that of the SH2 domain alone, since full-length Grb14 binds to FGFR1 but not to PDGFR. This suggests that studies on SH2 domain-containing adaptors that utilize the whole protein in binding assays may be more effective at identifying true in vivo binding partners.

In addition to the interaction with activated FGFR1, Grb14 has been shown to bind to the IR following treatment of cells with insulin (28). However, unlike the interaction with FGFR1, which involves both the juxtamembrane and C-tail domains of the receptor, Grb14 binding to the IR occurs within the kinase domain and appears to require neither the juxtamembrane nor the C-tail domain of the receptor. Grb10 also binds to the IR, although the sites of binding are less clear, since interactions with the C-tail (42) and the kinase and juxtamembrane domains (25) have been reported. Grb10 exists as multiple splice variants (7), and this may in part account for the different molecular interactions described. The interaction of Grb14 with both FGFR1 and the IR is consistent with the interaction of all Grb7 family members with multiple receptors and may indicate that the Grb7 family represents a point of convergence for the initiation of multiple signaling pathways.

The binding of Grb14 to other cellular proteins may be regulated by phosphorylation of Grb14. Grb14 is phosphorylated on serine and threonine residues but not on tyrosine residues and, therefore, is not a direct substrate of FGFR1. Treatment of cells with PDGF (27) or FGF-2 enhances the phosphorylation of Grb14, indicating the involvement of kinases downstream of these receptors. The role that phosphorylation of Grb14 plays in binding to target proteins remains to be elucidated, but it is possible that binding specificity is altered by phosphorylation, potentially restricting Grb14 to a subset of binding partners appropriate for the cellular stimulus.

The present study demonstrates a role for Grb14 in FGF-
induced cellular proliferation. Expression of exogenous Grb14 inhibited FGF-stimulated DNA synthesis, whereas expression of Grb14 R466K potentiated the effect of FGF-2 on proliferation. Grb14 has also been shown to inhibit insulin-induced proliferation in Chinese hamster ovary IR cells (28). The data in the present study are consistent with those results and support a negative regulatory role for endogenous Grb14 in cellular proliferation. The potentiation of FGF-induced proliferation by Grb14 R466K also provides evidence for a negative regulatory role of endogenous Grb14, although clarifying the mechanism of this effect will require further study. Since Grb14 R466K does not bind to FGFR1, this effect is most likely mediated by as yet unidentified downstream Grb14 binding partners. The mutant Grb14 may prevent the activation of downstream effectors in an inhibitory cascade normally tied into receptor activation by endogenous Grb14. Alternatively, the mutant Grb14 may form non-functional oligomers with endogenous Grb14. Oligomerization of Grb10 has been reported (43), and nonfunctional Grb14 oligomers containing the R466K point mutant may release cells from the inhibitory effects of the wild-type adaptor protein.

Grb10 is also involved in the regulation of cellular proliferation, although opposite effects have been observed in different systems. Splice variants of Grb10 have been shown to inhibit insulin-like growth factor I-stimulated proliferation (44) as well as insulin-induced phosphorylation of IR substrates (45). Other studies have suggested that Grb10 stimulates insulin-, insulin-like growth factor I-, and PDGF-induced mitogenesis (18, 46). Some of these studies utilized microinjection of SH2 domain fusion proteins. The data in the present study indicate that the isolated Grb14 SH2 domain is less specific than full-length Grb14 with respect to receptor targets, and if the same is true for other Grb7 family members, it may complicate interpretation of the effects of SH2 domain fusions. Furthermore, given the potential of Grb7 family members to interact with a wide variety of cellular proteins, members of this family may play different roles in growth factor signaling depending on cell type and context. Grb10, in addition to its role in the regulation of proliferation, has been implicated in the regulation of apoptosis (24). Transfection of cells with a mutant form of Grb10 equivalent to the Grb14 R466K mutation induced apoptosis. Compared with the potentiation of FGF-induced proliferation seen with Grb14 R466K in the present study, the ability of a similar Grb10 mutant to potentiate apoptosis seen with Grb14 is explained by observed differences in the binding profiles of mutant and wild-type adaptors. Nevertheless, the potentiation of apoptosis by Grb14 R466K is consistent with the observed decrease in the expression of Grb14 and the potential of mutant Grb10 to induce apoptosis is consistent with the potential for Grb7 family members to function in a wide variety of cellular processes.

In addition to an SH2 domain, Grb14 contains a PH domain and a proline-rich region with a consensus SH3 domain binding site. c-Abl has been shown to bind to Grb10 via its SH3 domain (18, 46). Some of these studies utilized microinjection of SH2 domain fusion proteins. The data in the present study indicate that the isolated Grb14 SH2 domain is less specific than full-length Grb14 with respect to receptor targets, and if the same is true for other Grb7 family members, it may complicate interpretation of the effects of SH2 domain fusions. Furthermore, given the potential of Grb7 family members to interact with a wide variety of cellular proteins, members of this family may play different roles in growth factor signaling depending on cell type and context. Grb10, in addition to its role in the regulation of proliferation, has been implicated in the regulation of apoptosis (24). Transfection of cells with a mutant form of Grb10 equivalent to the Grb14 R466K mutation induced apoptosis. Compared with the potentiation of FGF-induced proliferation seen with Grb14 R466K in the present study, the ability of a similar Grb10 mutant to induce apoptosis is consistent with the potential for Grb7 family members to function in a wide variety of cellular processes.
Association of Fibroblast Growth Factor Receptor 1 with the Adaptor Protein Grb14: CHARACTERIZATION OF A NEW RECEPTOR BINDING PARTNER

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