Inhibition of Basal FGF Receptor Signaling by Dimeric Grb2

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SUMMARY

Receptor tyrosine kinase activity is known to occur in the absence of extracellular stimuli. Importantly, this “background” level of receptor phosphorylation is insufficient to effect a downstream response, suggesting that strict controls are present and prohibit full activation. Here a mechanism is described in which control of FGFR2 activation is provided by the adaptor protein Grb2. Dimeric Grb2 binds to the C termini of two FGFR2 molecules. This heterotrimer is capable of a low-level receptor transphosphorylation, but C-terminal phosphorylation and recruitment of signaling proteins are sterically hindered. Upon stimulation, FGFR2 phosphorylates tyrosine residues on Grb2, promoting dissociation from the receptor and allowing full activation of downstream signaling. These observations establish a role for Grb2 as an active regulator of RTK signaling.

INTRODUCTION

Members of the fibroblast growth factor receptor (FGFR) family of receptor tyrosine kinases (RTKs) are key regulators of many cellular processes, including proliferation, cell-cycle control, migration, and differentiation (Turner and Grose, 2010). Aberrances in enzymatic activity of these receptors causes a variety of human cancers and developmental defects (Robertson et al., 2000; Pollock et al., 2007; Beenken and Mohammadi, 2009; Zhao et al., 2010; Wesche et al., 2011). FGFRs are composed of an extracellular region that consists of two or three immunoglobulin (Ig)-like domains, a transmembrane region, and an intracellular region that includes a tyrosine kinase domain and flanking regulatory polypeptide sequences. FGFR signaling is initiated by the binding of extracellular growth factors that are conjugated to cell surface heparin sulfate proteoglycans (HSPGs). On engaging the growth factor, the receptor dimerizes, resulting in upregulation of intrinsic kinase activity and subsequent autophosphorylation of specific tyrosine residues in the C termini. This provides sites for recruitment of proteins and activation of downstream signaling pathways such as the mitogen-activated protein (MAP) kinase pathway. The relationship between phosphorylation and signal transduction is not, however, quite that simple. Phosphorylation of FGFR2 prior to extracellular stimulation in numerous cell lines, even under conditions of serum starvation, has been widely observed (Man-sukhani et al., 2000; Takeda et al., 2007; Kunii et al., 2008; Bryant et al., 2009; Ahmed et al., 2010). Importantly this “background” level of phosphorylation experienced by the receptor is insufficient to activate a downstream response until growth factor stimulation occurs. Therefore, rather than being in a nonphosphorylated “ground state,” the receptor is, in effect, being held in a nonsignaling, phosphorylated state primed for upregulation and full activity. To ensure that this primed state is incapable of effecting aberrant signals, a strict negative control mechanism is required to prohibit full activation until FGFR2 is exposed to growth factor.

Numerous alternative gene splicing events dictate structural variants and, hence, function of FGFR2. Splicing of sequences encoding the C terminus provides a major group of FGFR2 isoforms. Variants that result in deletions of the C-terminal sequence show enhanced transforming activity (Cha et al., 2008). These variants are expressed in increased amounts in gastric cancer cell lines (Itoh et al., 1994; Ishii et al., 1995) and in a majority of human breast carcinoma cells (Cha et al., 2009). Recently, point mutations in the C-terminal region of FGFR2 have also been linked with melanoma (Gartsdie et al., 2009). This suggests an important role for the C terminus of FGFR2 in control of receptor activity.

It was recently reported that the C-SH3 domain of growth factor receptor-bound protein 2 (Grb2) binds to a proline-rich sequence at the very C terminus of FGFR2 prior to growth factor stimulation. Deletion of the last 10 residues of the receptor abrogated Grb2 binding and concomitantly downregulated the phosphorylation of FGFR2 in the basal state (Ahmed et al., 2010). Furthermore, basal phosphorylation is also compromised in cells expressing the oncogenic S252W mutation in FGFR2, which is unable to bind Grb2. Therefore, Grb2 is strongly implicated in controlling FGFR2 kinase activity prior to growth factor stimulation.

Grb2 is ubiquitously expressed and plays a role in several signaling pathways from RTKs. As an adaptor protein, it is generally thought to play a passive role in signal transduction,
that the phosphorylation of FGFR2 depends on intracellular conditions (Figure 1B). To demonstrate this, showing that basal FGFR2 phosphorylation is not concentration of pFGFR2 (pFGFR) was confirmed in HEK293T cells (Figure 1A). As the 2010). This basal, “nonstimulated” phosphorylation of FGFR2 phosphorylation. Grb2 Binding Is Required for Basal State Receptor Phosphorylation Several recent reports show the background level of FGFR2 phosphorylation of Grb2 and its concomitant release from FGFR2 in the absence of extracellular stimulus. Binding of Grb2 to the C terminus of FGFR2 regulates the activation of a downstream signaling response. We demonstrate that Grb2 is a novel target for FGFR2 and show that, on stimulation by FGF, the kinase activity of the receptor is upregulated, promoting the phosphorylation of Grb2 and its concomitant release from the receptor. This leaves FGFR2 free to effect downstream signaling.

RESULTS

Grb2 Binding Is Required for Basal State Receptor Phosphorylation Several recent reports show the background level of FGFR2 phosphorylation in serum-starved cells (e.g., Ahmed et al., 2010). This basal, “nonstimulated” phosphorylation of FGFR2 (pFGFR) was confirmed in HEK293T cells (Figure 1A). As the anti-pFGFR antibody targets two tyrosine residues (Y653 and Y654) in the kinase activation loop, our data suggest that these residues are phosphorylated, which is consistent with the kinase domain being in an active conformation. FGFR2 signals through the MAPK pathway, but, importantly, the observed background receptor phosphorylation was insufficient to provide an active response as indicated by the absence of phosphorylated ERK1/2 (pERK) prior to the addition of FGF9 (Figure 1A). To demonstrate that the basal phosphorylation of FGFR2 was not derived from extracellular stimuli that might be present even under serum starvation conditions (such as residual growth factors/cytokines or from interactions with proteoglycan components of the extracellular matrix), we blotted for FGFR2 phosphorylation using a truncated receptor in which the extracellular, ligand binding domain had been deleted (except for the membrane localization sequence at the very C terminus; FGFR2cyto residues 400–821) from Escherichia coli (see Extended Experimental Procedures). FGFR2cyto was then incubated for different time periods with ATP and Mg2+ in the presence or absence of Grb2 and immunoblotted against phosphotyrosine (pY) (Figure 1D). At the different time points, when Grb2 is present, the level of receptor phosphorylation is markedly enhanced compared to when it is absent (Figure 1D, inset).

Additional evidence for Grb2 binding being sufficient to enhance FGFR2 phosphorylation is provided by demonstrating that in vitro phosphorylation of the receptor is abrogated in the absence of a functional Grb2-binding site. To demonstrate that the binding of Grb2 was necessary for FGFR2 phosphorylation, we incubated the FGFR2 isolated kinase domain (which cannot recruit Grb2) or FGFR2cyto with Grb2 and ATP/Mg2+ and determined the extent of receptor phosphorylation in the presence of increasing concentrations of Grb2. The level of phosphorylation of the kinase domain is unaffected by the increasing concentration of Grb2 (Figure S1C), whereas increasing amounts of Grb2 enhance the pFGFR2cyto concentration (Figures S1D and S1E). This confirms that Grb2 binding is required for receptor phosphorylation.

The Grb2-FGFR2 Complex Involves an Extended Interface Although structural models for the apo-Grb2 C-SH3 have been reported (Kohda et al., 1994; Maignan et al., 1995), no solution nuclear magnetic resonance (NMR) spectral assignments have been deposited/published. To provide a structural description for the recognition of FGFR2 by Grb2, we used triple resonance experiments to assign the backbone amides and both Cα resonances (deposited in the Biological Magnetic Resonance Bank [BMRB]) and mapped these on to the solution structure of Grb2 C-SH3 (Protein Data Bank [PDB] ID: 1GFC). Complex formation was confirmed by chemical shift changes in the NMR resonances in the H1,15N HSQC spectra of 15N-labeled Grb2 C-SH3 upon addition of aliquots of the C-terminal 15 amino acids of FGFR2 (FGFR215 residues 807–821; Ac-PSPLOQYPHINGSVKT-NH2). The resonance shifts were gradual with negligible broadening effects, characteristic of a fast exchange regime on the NMR chemical shift time scale (typical of weak binding with Kd of the order of >10 μM). Changes in the weighted average chemical shifts over a threshold value of 0.01 ppm were observed for amide residues Y160, V161, F167, E171, R178, N192, W193, A197, M204, F205, R207, Y209, and the indole ring side chain NH of W193 (Figure S2A). The intermolecular nuclear Overhauser effects (NOEs) for the complex were difficult to determine due to the weak interaction; thus, we have previously reported that the C-terminal SH3 domain of Grb2 (C-SH3) is able to bind to an amino acid sequence at the very C terminus of FGFR2 (Ahmed et al., 2010). In cells expressing receptors in which the C-terminal Grb2-binding site is deleted (Ahmed et al., 2010), or in which Grb2 has been knocked down using shRNA, background phosphorylation is lost or severely reduced (Figure 1C). Subsequent extracellular stimulation of FGFR2 led to increased receptor phosphorylation. Thus, the presence of Grb2 is able to enhance receptor phosphorylation in the basal state. To demonstrate this in vitro, we expressed and purified recombinant dephosphorylated cytoplasmic region of FGFR2 (FGFR2cyto residues 400–821) from Escherichia coli (see Extended Experimental Procedures). FGFR2cyto was then incubated for different time periods with ATP and Mg2+ in the presence or absence of Grb2 and immunoblotted against phosphotyrosine (pY) (Figure 1D). At the different time points, when Grb2 is present, the level of receptor phosphorylation is markedly enhanced compared to when it is absent (Figure 1D, inset).
Figure 1. Basal Phosphorylation of FGFR2 Requires Grb2

(A) HEK293T cells stably expressing WT FGFR2 (GFP-tagged) were serum-starved overnight. Recombinant FGF9 (20 ng/ml) was used to stimulate cells for 15 min. Fifty micrograms of total cell lysate were used for immunoblotting studies. First panel: immunoblot probed with an anti-pFGFR2 antibody. Third panel: immunoblot probed with an anti-pERK antibody to show the activation levels of FGFR2 and ERK in the total cell lysates. Anti-FGFR2 antibody (second panel), anti-ERK1/2 antibody (fourth panel), and anti-tubulin antibody (fifth panel) were used to assess protein loading.

(B) HEK293T, FGFR2ΔD, and Wild-type FGFR2

(C) FGFR2-CI and FGFR2-Grb2

(D) Without Grb2 and Grb2

employed the high ambiguity driven docking (HADDOCK) program (see Extended Experimental Procedures) to model the structure of the complex. The backbone root-mean-square deviation (RMSD) of the apo- and peptide-docked Grb2 C-SH3 domains was 0.88 Å, suggesting that FGFR215 does not introduce large changes in backbone conformation of the SH3 domain. Based on the model, the N-terminal proline-rich segment of FGFR215 has an extended structure that adopts a left-handed PPII helical conformation of class II proline-rich ligands and fits into the canonical PXXPXR-binding site on C-SH3. Interestingly, these interactions appear to orientate FGFR2 Y812 of the peptide toward Y209 of Grb2 (Figure 2A). This juxtaposition of phosphorylatable residues has an important role in dissociation of the complex on receptor stimulation (see below). Our model shows that the C-terminal region of FGFR215 (I815-T821) forms a left-handed β-like loop structure that wraps around the RT loop of the domain with K820 and T821 curled on to the other side of the SH3 domain. This conformation is stabilized through electrostatic interactions between K820 and H814 with E171. This results in a large region around the RT loop appearing to form an unusual, additional binding pocket that has not been previously observed in Grb2 (Yuzawa et al., 2001) or other SH3 domain-proline-rich peptide complexes (Figures 2A and S2B–S2D). A comparison of this structure with other reported SH3 domain complexes is given in the Extended Experimental Procedures. This unusual binding

Figure 2. FGFR2 C-Terminal Binds to an Extent Interface on the Grb2 C-SH3 Domain, and the Full-Length Grb2 Forms a Stable Dimer

(A) Structural model of Grb2 C-SH3 domain bound to a 15-residue peptide from the FGFR2 very C terminus. The complex structure was modeled using the program HADDOCK. Left panel: surface representation of Grb2 C-SH3 domain (red) in complex with FGFR2 peptide (stick representation: carbon atoms in cyan), Y209 on the surface of C-SH3 is shown in green. Right panel: identical structure to that shown in left panel rotated by 180°. C-SH3 domain (shown in ribbons) and FGFR2 peptide (stick: cyan with proline residues in magenta). See also Figure S2.

(B) Plot of hydrodynamic radius as a function of concentration for dimeric Grb2 (red circles), monomeric FGFR2cyto (black squares) and the heterotetrameric complex formed by the mixture of FGFR2cyto:Grb2 (blue triangles) in a 1:1 molar ratio (for radius values, see Figures S3A and S3B and Tables S2 and S3). Error bars shown on the mean value of three independent experiments.

(C) Grb2 dissociation measured by MST. Unlabeled Grb2 protein (55 nM to 100 μM) was titrated into a fixed concentration of labeled Grb2 (100 nM). The top panel shows the raw data for thermophoresis recorded at 20°C using the red LED at 10% and IR-Laser at 40%. The bottom panel shows the isotherm derived from the raw data and fitted according to the law of mass action to yield an apparent KD,dimer (0.66 ± 0.20 μM).
Grb2 Induces FGFR2 Dimerization prior to Extracellular Stimulation

The full-length Grb2 is capable of forming a discrete dimer (Maignan et al., 1995; McDonald et al., 2008). The physiological relevance of this structure has been unclear; however, it has important mechanistic consequences in determining the control of receptor phosphorylation. Dynamic light scattering (DLS) performed over the concentration range 0.4–40 μM revealed that a discrete Grb2 dimer (dynamic radius ~3.1 nm) was the dominant species (Figure 2B). Below this concentration, the population of dimer diminished, resulting in monomeric protein. We were able to fit microscale thermophoresis (MST) at concentrations up to 100 μM to give an apparent equilibrium dimer dissociation constant, Kd,dimer ~0.7 μM (Figure 2C).

Because Grb2 is able to form a stable dimer, it is important to establish whether it was capable of forming a complex with FGFR2 in this self-associated state. We previously reported binding data for the interaction of Grb2 with peptides corresponding to the very C terminus of FGFR2 (Ahmed et al., 2010). A more physiologically relevant assessment of the interaction was provided using isothermal titration calorimetry (ITC) to measure the binding of Grb2 to intact FGFR2cyto (Figure 3A; Table S1). As FGFR2 (150 μM) was titrated into Grb2 (15 μM), an initial complex was formed with a stoichiometry of 2:1 Grb2:FGFR2 and apparent equilibrium dissociation constant, Kd, of 0.1 μM (schematic in Figure 3A). This interaction is approximately 40-fold tighter than the binding of Grb2 to the FGFR215 peptide (Ahmed et al., 2010), suggesting that additional interactions might be possible in the context of FGFR2cyto. As more receptor was titrated, the 2:1 complex became saturated by FGFR2, giving a final complex with a stoichiometry of unity. This second binding event has a weaker affinity (~25 μM). The physiological relevance of this apparent anticooperativity is unknown but could result from conformational change of the interacting molecules or steric hindrance on assembly of the complex in this in vitro experiment. Because Grb2 exists as a dimer under the concentration conditions used in the ITC experiment (see above) and binding to FGFR2 is only detected for the Grb2 C-SH3 domain, the ITC data can be explained by assuming that, as more FGFR2 is titrated into the 2:1 Grb2:FGFR2 complex, the unbound Grb2 C-SH3 domain binds an additional receptor molecule forming a 2:2 heterotetrameric complex (schematic in Figure 3A). DLS was used to confirm the in vitro assembly of the heterotetramer (Figures 2B and S3A–S3C). This complex is consistent with the Grb2 dimer binding to FGFR2 in a conformation in which the C-SH3 domains are exposed and accessible to the receptor as seen in the crystal structure of intact Grb2 (Maignan et al., 1995).

We have shown that Grb2 is essential for the observed phosphorylation of FGFR2 prior to stimulation. Grb2 also exists as an isolated dimer in solution, which forms a 2:2 heterotetrameric complex binds with FGFR2 under saturating concentrations of receptor. Thus, a model whereby Grb2 is able to recruit two receptor molecules to predimerize and effect restricted kinase activity in the absence of extracellular stimulus is appealing. To test this model, we used an in vitro fluorescence spectroscopic method based on the mixing of FGFR2cyto fused to either green or blue fluorescent protein (GFP or BFP, respectively). Excitation of BFP results in an emission spectrum that overlaps with the excitation wavelength of GFP. Thus, bringing the two fusion proteins into close proximity can produce fluorescence resonance energy transfer (FRET), resulting in GFP emission at 510 nm. The two FGFR2 samples (5 and 10 nM) were mixed and irradiated at 383 nm to excite the BFP donor. In the solution of BFP–FGFR2cyto and GFP–FGFR2cyto in the absence of Grb2, the fluorescence spectrum looks similar to that for BFP–FGFR2cyto alone, suggesting that the receptors are not colocalizing and thus no FRET signal is apparent (Figure 3C). On the addition of increasing concentrations of Grb2 (1 nM to 2 μM), the GFP emission signal at 510 nm increased up to approximately 0.5 μM (Figure 3C, inset top; raw data are reported in Figure S3D [note Grb2 Kd,dimer ~0.7 μM; see above]). Consistent with the aforementioned model of Grb2 dimer being capable of bringing FGFR2 receptor molecules together, these data show that, as the Grb2 concentration increases, an increase in FRET efficiency occurs as a result of an increase in the population of complexes involving GFP–FGFR2:BFP–FGFR2. This effect is mirrored in the FRET distance (R; Figure 3C, inset bottom).

Additionally, we demonstrated recruitment of Grb2 to FGFR2 in cells. We transiently transfected HEK293T cells (in the presence and absence of FGFR2 expression) with both GFP- and RFP-tagged Grb2. Using fluorescence lifetime imaging microscopy (FLIM), FRET associated with Grb2 self-association (expected to be dimer) was observed to be cytoplasmically dispersed throughout cells without FGFR2, but membrane localized in the presence of FGFR2 (Figures S4A and S4B).

Based on our model in which Grb2 dimers recruit receptors, adding an excess of Grb2 dimer to a preexisting heterotetramer will result in saturation of FGFR2 molecules by Grb2 dimers forming Grb2:FGFR2:2:1 complexes (schematic in Figure 4A). These complexes are inhibitory to Grb2-induced receptor dimerization and will result in a reduction in the FRET signal. Figure 4A shows that with the Grb2 concentration regime from 1–40 μM, the fluorescence acceptor signal increases up to ~10 μM but decreases dramatically as more Grb2 is added and the receptors are no longer capable of forming heterotetramers.

The in vitro experiments suggest that expression of high concentrations of Grb2 in cells will be inhibitory to background receptor phosphorylation. To demonstrate this in a cellular context, we used increasing concentrations of Grb2-containing plasmid (1 ng–5 μg) to induce different levels of Grb2 expression in serum-starved stable HEK293T cells expressing FGFR2 (Figure 4B, lower panel). At low concentrations of Grb2, the level of activation loop tyrosine phosphorylated FGFR2 (pFGFR2) was low. As the amount of Grb2 in the cell was increased, FGFR2 phosphorylation increased (e.g., see ~0–100 ng in Figure 4B, top panel, and 4C), consistent with more prestimulated receptor dimer formation. It is important to note that, in these unstimulated cells at increased Grb2 concentrations, although the basal background phosphorylation of FGFR2 increases, there is no apparent downstream signaling effected (as seen by lack of pERK; Figure 4B, middle panel). This only occurs once growth
factor is added. As even higher concentrations of Grb2 were expressed, the level of receptor phosphorylation was reduced (Figures 4B, top panel, and 4C). This is consistent with the aforementioned FRET data and the proposed model for Grb2 at high concentrations inhibiting the formation of Grb2:FGFR2 heterotetramers (schematic in Figure 4B). Because the inhibited complexes containing only one receptor are incapable of intermolecular kinase activity, background phosphorylation is abrogated (Figures 4B and 4C). Additional experiments show that this inhibitory effect of high Grb2 concentrations on receptor phosphorylation is not due to extracellular ligand binding (Figure S1A), the FGFR2 fusion tag (Figure S5A), or overexpression of FGFR2 (Figures S5B and S5C).

Phosphorylation of Grb2 by FGFR2 Abrogates Complex Formation

From the proposed NMR structural model, a potential mechanism for the release of Grb2 from FGFR2 is apparent. This model shows that juxtaposed within the binding interface are two tyrosine residues (Grb2 Y209 and FGFR2 Y812; Figure 2A). As the phosphorylation of Grb2 by FGFR2 Abrogates Complex Formation

From the proposed NMR structural model, a potential mechanism for the release of Grb2 from FGFR2 is apparent. This model shows that juxtaposed within the binding interface are two tyrosine residues (Grb2 Y209 and FGFR2 Y812; Figure 2A). As the
side chains of these residues come into close proximity, post-translational modification of these residues with large negatively charged phosphate groups would lead to a charge repulsion or steric clash and, hence, disruption of binding. Therefore, if upregulation of the receptor can phosphorylate Grb2 and/or FGFR2, this would provide a switch for the release of the control of the basal receptor activity. Grb2 has been shown to be phosphorylated by other tyrosine kinases. For example, Grb2 is a substrate for EGFR (Haines et al., 2009), and Bcr/Abl phosphorylates Grb2 SH3 domain tyrosines Y7, Y52, and Y209 (Li et al., 2001). We incubated increasing concentrations of both FGFRcyt and the isolated kinase domain of FGFR2 (FGFRkin) with Grb2 in the presence of ATP and Mg$_2^+$ for 15 min and blotted with an anti-pY antibody to assess phosphorylation of Grb2. Figure 5A shows that FGFRcyt, but not FGFRkin, was able to phosphorylate Grb2 in vitro. Thus, Grb2 is a substrate for FGFR2 kinase activity; furthermore, the C-terminal FGFR2 Grb2-binding site is required for phosphorylation to occur.

Grb2 has seven tyrosine residues that are potential substrates for FGFR2 kinase (Figure 5B). Truncated versions of Grb2 were made to confirm that tyrosine residues on the C-SH3 domain were capable of being phosphorylated by FGFR2. The N-SH3, SH2, and C-SH3 domains of Grb2 were exposed to FGFRcyt, ATP, and Mg$^{2+}$ on ice for 15 min. Total cell lysates (50 μg) were used for immunoblotting studies. Upper panel: immunoblot probed with an anti-pFGFR2 antibody shows increasing concentration of pFGFR2 as the transfected Grb2 DNA increases to 100 ng. After this Grb2 concentration, the level of pFGFR2 decreases. This decrease in cellular pFGFR2 results from the same mass action effects as seen in Figure 4A whereby increasing concentrations of Grb2 protein expressed form more 2:1 Grb2:FGFR2 complex in the presence of excess Grb2. This is inhibitory to FGFR2 dimerization and, hence, phosphorylation.

To determine whether FGFR2 was capable of Grb2 phosphorylation in a cellular context, HEK293T cells stably expressing GFP-FGFR2 were transiently transfected with 1 ng–5 μg plasmid containing WT Grb2 DNA as indicated. Cells were serum-starved overnight. FGF9 (20 ng/ml) was used to stimulate thestarved cells for 15 min. Total cell lysates (50 μg) were used for immunoblotting studies. Upper panel: immunoblot probed with an anti-pFGFR2 antibody shows increasing concentration of pFGFR2 as the transfected Grb2 DNA increases to 100 ng. After this Grb2 concentration, the level of pFGFR2 decreases. This decrease in cellular pFGFR2 results from the same mass action effects as seen in Figure 4A whereby increasing concentrations of Grb2 protein expressed form more 2:1 Grb2:FGFR2 complex, which inhibit dimerization and, hence, phosphorylation of the receptor (see schematic). See also Figures S4 and S5.

(C) Graphical representation of the data shown in Figure 4B. Densitometric data for the pFGFR2, normalized against total FGFR shows the concentration of pFGFR2 reaches a maximum as the cellular Grb2 concentration is increased. The reduction in pFGFR2 results from the accumulation of 2:1 Grb2:FGFR2 complex in the presence of excess Grb2. This is inhibitory to FGFR2 dimerization and, hence, phosphorylation.
Finally, we used ITC to assess the binding between full-length phosphorylated Grb2 (pGrb2) and pFGFR2cyto to demonstrate that, in the phosphorylated state, the proteins were unable to interact. Incubation of both proteins for 1 hr in the presence of ATP and Mg$^{2+}$ ensured that they were phosphorylated. This was confirmed by mass spectroscopic analysis. No binding was discernable for pFGFR2 and pGrb2 by ITC under the conditions previously used to measure binding between the

Figure 5. Grb2 Is Phosphorylated by FGFR2
(A) Both purified FGFR2cyto (residues 400–821) and FGFR2kin (residues 412–760) domains were tested for their effects on Grb2 phosphorylation. Different amounts (0.01–4 nM) of FGFR2cyto and FGFR2kin were mixed with 500 ng of Grb2 protein with or without ATP/MgCl$_2$ in a 100 μl solution. Reactions were incubated on ice for 15 min and samples were denatured and analyzed using immunoblotting. The data show that, although FGFR2cyto can phosphorylate Grb2 (see lanes 6 and 7), under the same conditions FGFR2kin cannot (lanes 13 and 14). This suggests that a direct interaction via the SH3 domain of Grb2 and the FGFR2 C-terminal tail is required for Grb2 phosphorylation. The results were analyzed by immunoblotting with an anti-pY antibody (top panel) and an anti-Grb2 antibody as the Grb2 loading control (bottom panel).

(B) Top: Schematic representation of Grb2 and truncated forms thereof used to identify phosphorylated tyrosine residues. The tyrosine residues are numbered and shown as green circles. Bottom (immunoblot): Phosphorylation of Grb2 by FGFR2 in vitro. Purified FGFR2cyto and the Grb2 N-SH3, SH2 and C-SH3 domains were used to identify the phosphorylation status of Grb2. The FGFR2cyto (4 nM) was mixed with 200 ng of the individual domains of Grb2 with or without ATP and Mg$^{2+}$ in a 100 μl solution. Reactions were incubated on ice for 15 min, and samples were denatured and analyzed using immunoblotting. Both the N-SH3 and C-SH3 domains can be phosphorylated by FGFR2 in 15 min in the presence of ATP/Mg$^{2+}$ (middle panel, lanes 6 and 8), whereas the SH2 domain is not tyrosine phosphorylated (middle panel, lane 7). The results were analyzed by immunoblotting with an anti-pY antibody (top and middle panels). The loading control was carried out with Ponceau staining in the absence of a suitable antibody (bottom panel).

(C) Cellular phosphorylation of Grb2. HEK293T cells were cotransfected with FGFR2 and Grb2 (Strep-tagged) or vector and Grb2 (Strep-tagged), starved overnight, and then stimulated by FGF9 for 5 min. Cells were then lysed in the presence of protease and phosphatase inhibitors. Total cell lysate (50 μg) were used for immunoblotting studies. Total cell lysate (1 mg) were used for immunoprecipitation studies. The data show clear phosphorylation of Grb2 as revealed by anti-pY antibody. Left top panel (whole-cell lysate study): The expression of FGFR2 was confirmed using an anti-FGFR2 antibody. Lower panel: Grb2 from the FGFR2-expressing cells appears as at least two bands (arrows 1 and 2), suggesting that the higher mobility species (arrow 1) is/are tyrosine-phosphorylated Grb2.

(D) HEK293T cells stably expressing FGFR2-GFP were transiently transfected with 8 μg of WT, all 7F, Y7F, Y17F, Y52F, Y118F, Y134F, Y160F, Y209F, and Y7/52/160F mutated Grb2 DNA as indicated. Cells were serum-starved for overnight, FGF9 (20 ng/ml) was used to stimulate the starved cells for 15 min. Total cell lysates (50 μg) were used for immunoblotting studies. The immunoblot was probed with an anti-pFGFR2 antibody (top panel) and, with an anti-pERK antibody (central panel) to show the activation levels of FGFR2 and ERK. Anti-Grb2 blot (bottom panel) shows the overexpression of the various Grb2 constructs. Anti-tubulin antibody was used to confirm equal loading (central panel).
unphosphorylated polypeptides (Figure 3B). This is therefore consistent with a mechanism whereby phosphorylation of Grb2 releases it from the receptor.

According to the proposed mechanism, a Grb2 mutation Y209F should preclude the release of Grb2 from the extracellularly stimulated receptor, as phenylalanine cannot be phosphorylated by FGFR2 and, hence, no repulsive interaction will occur. To measure the effect of Y-F substitutions on Grb2, we mutated all of the available tyrosine residues on Grb2, either independently or in selected groups. These Grb2 mutants were transfected into stable HEK293T cells overexpressing WT FGFR2. Stimulation of the receptor by FGF9 shows a pronounced effect on receptor phosphorylation in the presence of one of the mutants (Figure 5D). In the two cell lines in which Y209F was present (i.e., “all 7F” and “Y209F”), a clear phenotype was revealed in which poststimulatory phosphorylation of FGFR2 was inhibited. However, the Y209F mutant still showed the basal phosphorylation, indicating that the mutant did not abrogate Grb2 binding to FGFR2. This difference between pre- and poststimulation pFGFR2 concentrations is not apparent in the context of the other Y-to-F mutations, and the bands are similar to that seen for the WT Grb2. These results show that, if Grb2 is unable to be removed from the receptor, then the ability of the receptor to phosphorylate itself (either cis or trans) in the stimulated state is strongly affected. This is likely to be in part due to Grb2 sterically hindering FGFR2 C-terminal recruitment to the kinase domain and thus inhibiting access to the tyrosine residues.

DISCUSSION

Phosphorylation of tyrosine residues on an RTK provides sites for recruitment of signaling proteins and, under appropriate conditions, transduction of a downstream response. Receptor activity in the absence of extracellular stimulation suggests that control of the phosphorylation is fundamental to cellular function and survival. RTKs exhibit idiosyncratic modes of basal kinase inhibition, stimulant recognition, and upregulation of kinase activity (Zhang et al., 2006; Lemmon and Schlessinger, 2010). Here we report a novel control mechanism in which FGFR2 is held in a dimeric state by binding of a Grb2 dimer at the very C terminus of the receptor (see schematic model in Figures 6A–6C). In this heterotetrameric state, the receptor is capable of a low level of kinase activity but prohibited from effecting a downstream MAP kinase response. In addition, the binding of Grb2 in the largely unstructured C-terminal region of FGFR2 inhibits access to tyrosine residues that, on phosphorylation, form binding sites for recruitment of downstream signaling proteins. When the receptor is stimulated by a growth factor, the kinase activity is upregulated resulting in phosphorylation of Grb2 Y209. In its phosphorylated state, Grb2 is no longer capable of binding FGFR2. The liberated receptor can then undergo the required conformational changes to achieve a signaling-competent phosphorylated state. The structural basis of the complex of full-length Grb2 bound to the cytoplasmic region of FGFR2 has yet to be elucidated; however, based on our knowledge, a model can be constructed (Figure S6).

This novel regulatory mechanism is dependent on the ability of Grb2 to bind via its C-terminal SH3 domain to the C terminus of FGFR2, its potential to dimerize under cellular conditions ($K_{d \text{dimer}} \sim 0.7 \mu M$), and its ability to be phosphorylated by activated FGFR2 and, hence, released from the receptor. Whether this mechanism of RTK predimerization through Grb2 is unique to FGFR2 remains unclear; however, several other receptors have sequences that could form binding sites for Grb2 C-SH3 domain. For example, insulin receptor, insulinlike growth factor receptor, and epidermal growth factor receptors all have cognate SH3 domain-binding PXXP motifs in their C termini. Furthermore, SH3 domains are quite promiscuous in their binding, and recent screening studies have demonstrated that Grb2 C-SH3 is able to bind sequences found on RTKs as diverse as R/KXXR/K with micromolar affinity (Jia et al., 2005). Thus, it is tempting to speculate that FGFR2 is not the only receptor for which Grb2-mediated dimerization occurs.

The involvement of Grb2 in FGFR2 signal regulation provides a further variation on the theme of RTK regulation (Lemmon and Schlessinger, 2010); however, uniquely in this case, a third party adds a novel regulatory layer to the system formed by receptor and growth factor (excluding the involvement of cell matrix proteoglycans). There are examples where receptors and signaling molecules are recruited and assembled through adaptor protein binding; however, these occur as a consequence of ligand-induced receptor activation (Hu et al., 2003; Houtman et al. 2006). The role of Grb2 appears to be twofold: dimerization
of FGFR2 and controlling the phosphorylation so that FGFR2 is incapable of a MAPK downstream response. Predimerization of the receptor facilitates growth factor/HSPG recognition and binding, and, hence, lower concentrations of extracellular stimuli are required. Prephosphorylation of kinase domain tyrosines means that the receptor does not start at a zero-phosphorylated ground state, which is likely to have consequencies both energetically and with respect to the integrity of the order in which tyrosine residues are phosphorylated (Furdui et al., 2006).

Grb2-mediated basal control of FGFR2 signaling provides a dramatic alternative to the canonical view of RTK signaling and control thereof. The involvement of an intracellular protein in facilitating RTK dimerization and exerting control over kinase activity suggests that tyrosine kinase-mediated signal transduction cannot be represented by otherwise random coming together of receptors diffusing through the plasma membrane being locked into dimeric (or higher order) complexes in the presence of growth factors. In the case of FGFR2, this process is subject to additional influence. Furthermore, the role outlined for Grb2 elevates it from a passive protein simply linking others in signal transduction to an active regulator of receptor activity and potential suppressor of aberrant signals that can lead to cancer.

**EXPERIMENTAL PROCEDURES**

**Cells**

Human embryonic kidney 293T (HEK293T) cells and HEK293T cells expressing WT FGFR2 were maintained and grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 0.05 mg/ml gentamycin sulfate (Cambrex), and 1% Pen/Strep Fungizone Mixture (Cambrex) at 37°C in a 5% CO₂ humidified incubator.

**Molecular Cloning**

Gene fragments encoding FGFR2 and Grb2 constructs were amplified using PCR and were cloned into pGEX4T1 and pET33b vectors. Bacterial GFP/BFP expression vectors were generated on the pET28b vector backbone. Gene fragments encoding FGFR2 and Grb2 constructs were amplified using PCR and were cloned into pGEX4T1 and pET33b vectors. Bacterial GFP/BFP expression vectors were generated on the pET28b vector backbone. The extracellular domain-deletion FGFR2 construct was cloned into the BFP expression vectors were generated on the pET28b vector backbone. Gene fragments encoding FGFR2 and Grb2 constructs were amplified using PCR and were cloned into pGEX4T1 and pET33b vectors. Bacterial GFP/BFP expression vectors were generated on the pET28b vector backbone.

**Protein Expression and Purification**

6x histidine- and glutathione S-transferase (GST)-tagged constructs of FGFR2 and Grb2 were expressed and purified from *E. coli* as described in the Extended Experimental Procedures and by Ahmed et al. (2010).

**ITC**

ITC experiments were carried out using a VP Microcal instrument (Northampton, MA), and data were analyzed using ORIGIN7 software (Extended Experimental Procedures).

**MST**

The microscale thermophoresis (MST) method has been described in detail elsewhere (Jubbeke-Willemsen et al., 2011). The K_{D,therm} for Grb2 dimers was measured using the Monolith NT.115 from Nanotemper Technologies. Proteins were fluorescently labeled according to the manufacturer’s protocol. Labeling efficiency was determined to be 2:1 (protein to dye) by measuring the absorbance at 280 nm and 650 nm. A solution of unlabeled Grb2 was serially diluted from about 100 μM to 55 nM in the presence of 100 nM labeled Grb2. The samples were loaded into silica capillaries (Polymicro Technologies) after incubation at room temperature for 1 hr. Measurements were performed at 20°C in 20 mM HEPES buffer, pH 8.0, with 200 mM NaCl, 1 mM IME, and 0.05% Tween 20, by using 10% LED power and 40% IR-laser power. Measurements were also carried out on 35% LED power and 20% IR-Laser power for comparison. Data analyses were performed using Nanotemper Analysis software, v.1.2.101.

**DLS**

DLS experiments were performed at 20°C in a Wyatt Technologies DynaPro Titan instrument. Experiments were carried out in standard PBS buffer. Grb2 samples were centrifuged at 16,100 x g for 1 hr at 20°C to degas and remove any residual aggregates. DLS data were collected and analyzed using DYNAMICS V6.7.6 software for the DynaPro Titan instrument (Wyatt Technology Corporation).

**Fluorescence Spectroscopy**

Steady-state fluorescence was measured in triplicate at 15°C in 20 mM Tris-HCl, pH 8.0, with 50 mM NaCl and 1 mM IME using the QuantaMaster-4-CW from Photon Technology International. Spectra were measured from 400 to 550 nm with excitation at 383 nm. Excitation and emission bandwidths were set to be 0.5 nm. The emission of 10 nM BFP-FGFR2_{cyto} (donor) was measured followed by a second measurement performed in the presence of 5 nM GFP-FGFR2_{cyto} (acceptor). In the absence of Grb2, a small FRET signal was observed. Grb2 was added to BFP/GFP-FGFR2_{cyto} (which was kept at constant 2:1 molar ratio). FRET was monitored as the Grb2 concentration was increased from 1 nM to 2 μM. The Förster radius (R0), FRET efficiency (E), and distance (R) were calculated using the software PTI Felix32 Analysis, version 1.2, assuming that R_0 is 2/3 (fast rotation limit), the refractive index of water (n) is 1.33, quantum yields of 0.26, and extinction coefficient, ε ( l ), of 31,000 M⁻¹ cm⁻¹ at the wavelength of 383 nm. R_0 was calculated to be 27.4 ± 1.5 Å. Errors for E and R were estimated to be about 5%.

**NMR Spectroscopy**

The NMR experiments were performed on Bruker Avance III 700 MHz spectrometer equipped with a cryoprobe. All experiments were performed at 25°C as described in the Extended Experimental Procedures. All NMR data were processed using NMRpipe (Delaglio et al., 1995) and Azara software (Wayne Boucher, Department of Biochemistry, University of Cambridge, Cambridge, UK). The two- and three-dimensional (2D and 3D, respectively) NMR data visualization and analyses were performed with the CCPN Analysis program suite (http://www.ccpn.ac.uk/). Details on sequential assignment of backbone residues of the human Grb2 C-SH3 (residues 156–217) and of the HADDOCK and FlexPepDock docking methods are given in the Extended Experimental Procedures.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Extended Experimental Procedures, six figures, and three tables and can be found with this article online at doi:10.1016/j.cell.2012.04.033.

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