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# The Docking Protein FRS2α Controls a MAP Kinase-Mediated Negative Feedback Mechanism for Signaling by FGF Receptors

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## Summary

The docking protein FRS2α functions as a major mediator of signaling by FGF and NGF receptors. Here we demonstrate that, in addition to tyrosine phosphorylation, FRS2α is phosphorylated by MAP kinase on multiple threonine residues in response to FGF stimulation or by insulin, EGF, and PDGF, extracellular stimuli that do not induce tyrosine phosphorylation of FRS2α. Prevention of FRS2α threonine phosphorylation results in constitutive tyrosine phosphorylation of FRS2α in unstimulated cells and enhanced tyrosine phosphorylation of FRS2α, MAPK stimulation, cell migration, and proliferation in FGF-stimulated cells. Expression of an FRS2α mutant deficient in MAPK phosphorylation sites induces anchorage-independent cell growth and colony formation in soft agar. These experiments reveal a novel MAPK-mediated, negative feedback mechanism for control of signaling pathways that are dependent on FRS2 and a mechanism for heterologous control of signaling via FGF receptors.

## Introduction

Fibroblast growth factors (FGFs) constitute a large family of growth factors that play important roles in multiple cellular processes critical for the normal functions of many tissues in both invertebrates and vertebrates (reviewed in Goldfarb, 1996). Genetic studies demonstrated the crucial role of FGFs during the early stages of embryogenesis, when embryonic cells depend on FGF signaling for gene expression, morphological identity, migration, and axis development (Arman et al., 1998; Beddington and Robertson, 1999; Ciruna et al., 1997; Crossley and Martin, 1995; Deng et al., 1994; Feldman et al., 1995; Naski and Ornitz, 1998; Niswander and Martin, 1992; Sun et al., 1999; Yamaguchi et al., 1994).

FGFs mediate their pleiotropic responses by binding to and activating a family of receptor tyrosine kinases (RTKs) designated FGF receptors (FGFR) 1–4 (reviewed in Schlessinger, 2000). Many of the cellular responses of FGFs are mediated by the membrane-linked docking proteins, FRS2α and FRS2β (Kouhara et al., 1997; Xu et al., 1998; Hadari et al., 1998, 2001). Both FRS2α and FRS2β contain myristyl anchors and phosphotyrosine binding (PTB) domains in their N termini and multiple tyrosine phosphorylation sites in their C termini that serve as binding sites for the adaptor protein Grb2 and the protein tyrosine phosphatase Shp2 (Kouhara et al., 1997; Hadari et al., 1998). Although both FRS2 family members are highly homologous, their differential pattern of expression points to specific roles for each member. While FRS2α is ubiquitously expressed and can be detected at every developmental stage of the mouse, the expression of FRS2β begins at day 9 to 9.5 and is primarily confined to tissues of neuronal origin (McDougall et al., 2001; N. Gotoh et al., unpublished data). Indeed, the nerve growth factor (NGF) and glial cell line-derived neurotrophic factor (GDNF) families of neurotrophins also signal through FRS2α and FRS2β and regulate the activity of FRS2-dependent signaling pathways (Kurokawa et al., 2001; Melillo et al., 2001).

We have demonstrated that disruption of the FRS2α gene results in embryonic lethality at approximately E 7–7.5 due to severe defects in gastrulation (Hadari et al., 2001; our unpublished data). Experiments using FRS2α-deficient mouse embryo fibroblasts (MEFs) and MEFs reconstituted with either wild-type or various FRS2α mutants revealed that FRS2α (1) links the activated FGFR with the Ras-MAPK cascade, (2) has a critical role in FGF-dependent cell proliferation and migration, and (3) functions as a focus of assembly of a multiprotein complex that controls the Ras-MAPK cascade and the PI-3 kinase-dependent survival pathway. Upon FGF or NGF stimulation, FRS2α recruits four Grb2 molecules directly and two indirectly via Shp2 (Hadari et al., 1998). Rescue experiments using various FRS2α mutants revealed the differential importance of Grb2 and Shp2 binding sites in mediating FRS2α-dependent responses. In addition to its role in recruitment of the nucleotide exchange factor Sos by tyrosine phosphorylated FRS2α, Grb2 functions as a link between FRS2α and the docking protein Gab1. FGF stimulation leads to Gab1 recruitment, which is followed by recruitment of PI-3 kinase and activation of a cell survival pathway (Ong et al., 2001; Hadari et al., 2001). The Shp2 binding sites play a primary role in FGF-induced activation of MAPK, cell proliferation, and cell migration (Hadari et al., 2001). The Grb2 binding sites of FRS2α are essential for activation of PI-3 kinase (Ong et al., 2001) and the ubiquitin ligase Cbl (Wong et al., 2002) and have a secondary role in MAPK stimulation (Hadari et al., 2001).

In this report we demonstrate that in addition to enhancement of tyrosine phosphorylation, FGF stimulation induces MAP kinase-dependent phosphorylation of FRS2α on at least eight threonine residues resulting in a large shift in its electrophoretic mobility. Threonine phosphorylation of FRS2α is accompanied by reduced tyrosine phosphorylation of the docking protein, decreased recruitment of Grb2, and attenuation of the MAP kinase response. A similar FRS2α threonine phosphorylation is induced in response to PDGF, insulin, or EGF treatments, growth factors that do not induce tyrosine phosphorylation of FRS2α and do not stimulate the bio-

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logical responses of FGFs. These experiments demonstrate that in addition to its major role as a mediator of signaling via FGFRs, FRS2α participates in a negative feedback mechanism and an interreceptor control mechanism for regulation of FGF-receptor signaling.

## Results

We have previously demonstrated that in addition to tyrosine phosphorylation, FRS2α undergoes an electrophoretic mobility shift that is probably caused by phosphorylation of FRS2α on serine/threonine residues in response to FGF stimulation (Kouhara et al., 1997; Figure 1A). Indeed, the FGF-induced electrophoretic mobility shift was reversed by treatment of FRS2α immunoprecipitates with alkaline phosphatase (Figure 1A). In contrast, treatment of the cells with Okadaic acid, a protein phosphatase inhibitor, induced a pronounced electrophoretic mobility shift of FRS2α that is stronger than that induced by FGF stimulation (Figure 1A). Taken together, these experiments suggest that the shift in the electrophoretic mobility of FRS2α induced by FGF stimulation is caused by protein phosphorylation.

### Electrophoretic Mobility Shift of FRS2α Is Caused Primarily by MAPK-Mediated Phosphorylation of FRS2α

To examine the possibility that the mobility shift of FRS2α is caused by kinase(s) lying downstream of Ras, we have examined the effect of overexpression of a dominant interfering mutant of Ras-Ras N-17 (Ras DN) on FGF stimulation of FRS2α electrophoretic mobility shift. The experiment presented in Figure 1B shows that expression of Ras DN almost entirely prevents the mobility shift of FRS2α induced by FGF stimulation. The expression of Ras DN also inhibited the activation of MAPK in FGF-stimulated cells (Figure 1B) and correspondingly increased the tyrosine phosphorylation of FRS2α and its interaction with Grb2, even in unstimulated cells (Figure 1B). The effect of Ras-DN on the mobility shift of FRS2α induced by FGF stimulation was incomplete because tyrosine phosphorylation also causes a small, but detectable, electrophoretic mobility shift of FRS2α. Based on these results, we propose that the bulk of the electrophoretic mobility shift of FRS2α occurs as a result of phosphorylation by FGF-induced protein kinase(s) that lie downstream of Ras.

Sequence analysis revealed the presence of eight canonical MAPK phosphorylation sites (PXTP motifs) in FRS2α that are clustered in three separate regions of the docking protein (Figure 2A). One cluster containing three potential MAPK phosphorylation sites (PRTPRTPTTP) lies between amino acids 130 to 139 in a region that links the PTB domain to the rest of the protein. The second cluster (amino acids 374-377) containing a single potential MAPK phosphorylation site (PKTP) is located toward the C terminus of the protein. The third cluster containing four potential MAPK phosphorylation sites (amino acids 450-464, PQTPKTPTTPLPQTP) is flanked by the two Shp-2 binding sites in the C terminus of FRS2α. To study their functional role in FRS2α, we have replaced the threonine residues of the PXTP clusters with valine residues using site-directed mutagenesis in various combinations (each cluster is numbered according to its order of appearance along the FRS2α molecule), or all eight combined (FRS2α-8V). The effect of the substitutions on the electrophoretic mobility of the FRS2α mutants was determined by SDS-PAGE analysis of FRS2α immunoprecipitates isolated from lysates of unstimulated or FGF stimulated 293 cells expressing either wild-type

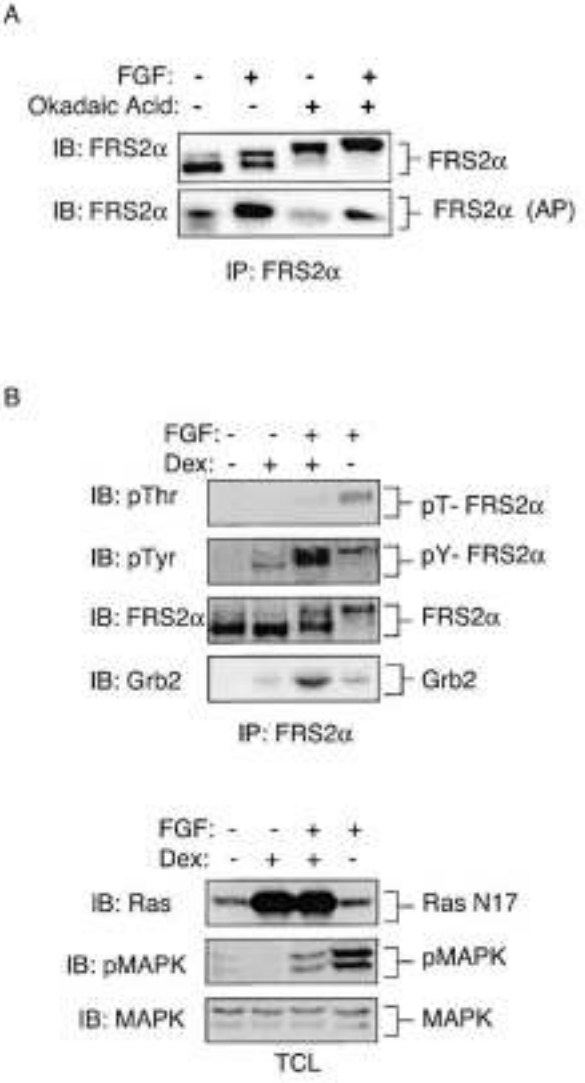



**Figure 1. FGF-Induced Electrophoretic Mobility Shift of FRS2α Is Mediated by Kinase(s) Dependent upon Ras Activation**

(A) FRS2α $^{-}$ MEFs expressing wild-type FRS2 were either left untreated or treated overnight with 120 nM okadaic acid. Lysates from unstimulated or FGF-stimulated cells were subjected to immunoprecipitation with anti-FRS2α antibodies. One-half of the sample was analyzed by SDS-PAGE, while the second half was incubated with 5 μg/sample of alkaline-phosphatase (AP) for 1 hr at 37°C before SDS-PAGE analysis, and both samples were subjected to immunoblotting with anti-FRS2α antibodies.

(B) PC12 cells transfected with a dexamethasone-induced Ras N-17 vector were left untreated or were treated overnight with 2 μM dexamethasone (Dex). Lysates from unstimulated or FGF-stimulated cells were immunoprecipitated with anti-FRS2α antibodies followed by SDS-PAGE and immunoblotting with various antibodies as indicated. Also shown, immunoblots with anti-Ras, anti-MAPK, and anti-p-MAPK antibodies of total cell lysates (TCL).

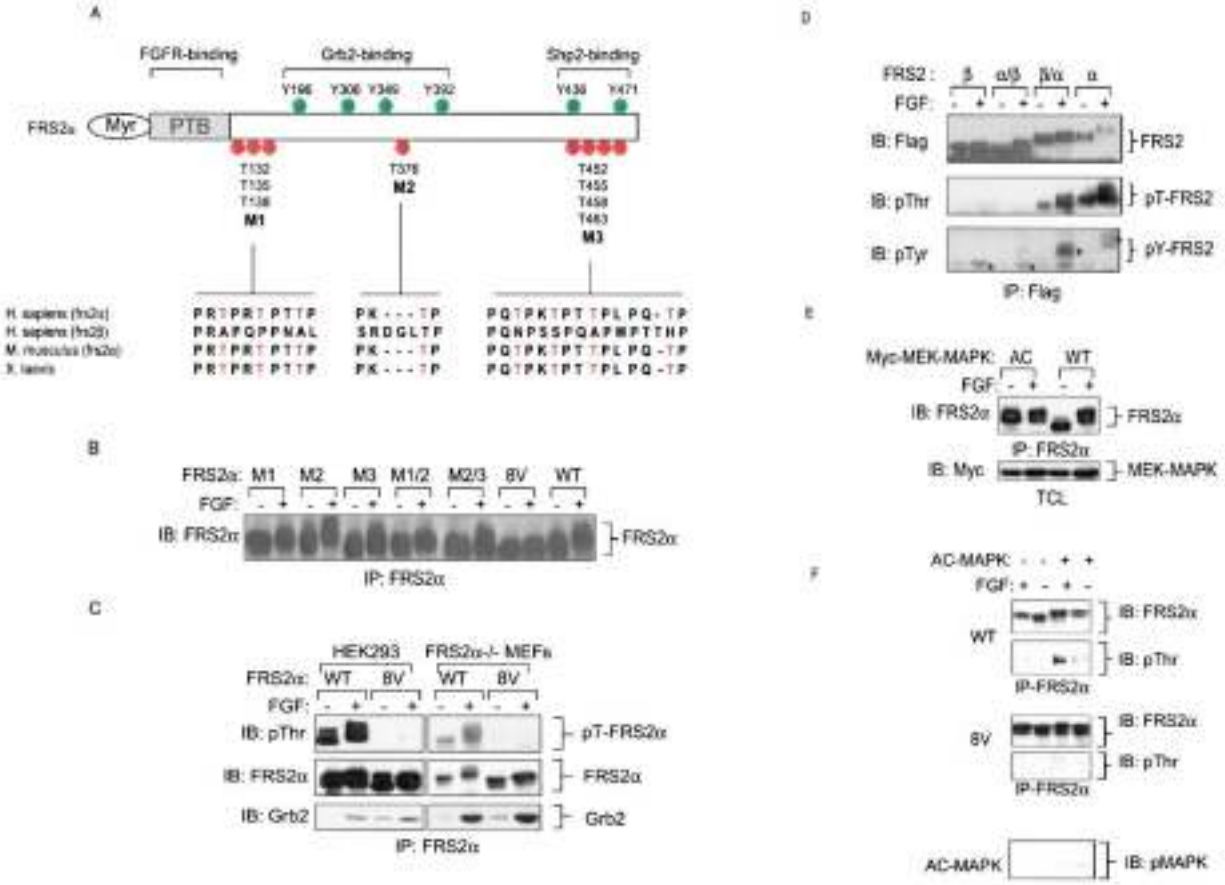



**Figure 2. The Electrophoretic Mobility Shift Is Caused by Phosphorylation of Eight Threonine Residues on FRS2α by MAPK**

(A) A schematic presentation of mouse FRS2α including the tyrosine phosphorylation sites and the putative MAPK threonine (Thr) phosphorylation sites. Also shown are comparisons of the amino acid sequences of the three clusters containing the putative threonine phosphorylation sites in human, mouse, and frog FRS2α, as well as the corresponding sequence in human FRS2β.

(B) HEK 293 cells were transiently transfected with either FRS2α-WT or various FRS2α mutants in which the putative MAPK threonine phosphorylation sites in the three separate clusters were mutated individually to valines (M1, M2, M3) or all combined (8V). Lysates from unstimulated or FGF-stimulated cells were subjected to immunoprecipitation with anti-FRS2α antibodies followed by immunoblotting with anti-FRS2α antibodies.

(C) HEK 293 cells transfected with FRS2α-WT or with the FRS2α-8V mutant and FRS2α$^{-/-}$ MEFs expressing FRS2α-WT or the FRS2α-8V mutant were stimulated with FGF or were left unstimulated. FRS2α immunoprecipitates were immunoblotted with anti-p-Thr, anti-FRS2α, and anti-Grb2 antibodies.

(D) HEK 293 cells were transfected with the following FLAG-tagged proteins: wild-type FRS2α (α), wild-type FRS2β (β), a chimeric protein in which the myristolation signal and the PTB domain of FRS2α were attached to the tail of FRS2β (FRS2α/β) and a chimera composed of the myristolation signal and PTB domain of FRS2β linked to the tail of FRS2α (FRS2β/α). Lysates of unstimulated or FGF-stimulated cells were immunoprecipitated with anti-FLAG antibodies followed by immunoblotting with anti-FLAG, anti-p-Thr, or anti-p-Tyr antibodies.

(E) HEK 293 cells were cotransfected with FRS2α and with either an expression vector for activated Myc tagged MEK-MAPK (AC) or with expression vector for wild-type Myc-MEK-MAPK (WT). Cell lysates of unstimulated or FGF-stimulated cells were first immunoprecipitated and then immunoblotted with anti- FRS2α antibodies. Also shown is an anti-Myc-tagg immunoblot of total cell lysates (TCL), to reveal the expression level of the activated and wild-type MEK-MAPK proteins.

(F) In vitro phosphorylation of FRS2α by purified activated MAPK. FRS2α and the FRS2α-8V mutant were immunoprecipitated from unstimulated or FGF-stimulated MEFs. The immunoprecipitates were washed once with a kinase buffer and then either left untreated or incubated with activated ERK2 for 30 min at 30°C. The samples were analyzed by immunoblotting with anti-pThr or anti-FRS2α antibodies. Activated ERK2 was immunoblotted with anti-p-MAPK antibodies.

or various FRS2α mutants. Only mutation of all eight putative MAPK phosphorylation sites in the three clusters prevented the electrophoretic mobility shift of FRS2α induced by FGF stimulation (Figure 2B). Similar results were obtained when all eight threonines were substituted by alanine residues (data not shown). In addition, by using antibodies raised against the canonical MAP kinase p-Thr sites (anti-p-Thr antibodies) for immunoblotting anti-FRS2α immunoprecipitates from lysates of 293 cells or from lysates of FRS2α$^{-/-}$ MEFs expressing either FRS2α-WT or the FRS2α-8V mutant, we demonstrated that FGF stimulation enhances threonine phosphorylation of FRS2α-WT but not threonine phosphorylation of FRS2α-8V (Figure 2C).

In order to shed further light on the role played by the three threonine-rich clusters in mediating the cellular

response of FRS2α, we have generated chimeric FRS2 proteins in which the carboxy termini of FRS2α (contains 8 PXpTP sites) and FRS2β (lacks consensus MAPK phosphorylation sites) were swapped. The chimeric FRS2 proteins are designated FRS2α/β or FRS2β/α. Next, 293 cells were transfected with expression vectors that direct the synthesis of FGFR1 together with expression vectors for FLAG-tagged FRS2α, FRS2β, FRS2α/β, or FRS2β/α. The experiment presented in Figure 2D depicts SDS-PAGE analysis of lysates from unstimulated or FGF stimulated cells that were subjected to immunoprecipitation with anti-FLAG antibodies followed by immunoblotting with either anti-FLAG or anti-p-Thr antibodies. This experiment demonstrates that endowment of the amino terminus of FRS2β with the carboxy terminus of FRS2α enables the molecule to become threonine phosphorylated and its mobility shifted in SDS-PAGE. By contrast, the FRS2α/β protein was not phosphorylated on threonine residues nor was the electrophoretic mobility of the chimeric protein altered in response to FGF stimulation. The integrity of both chimeric FRS2α/β and FRS2β/α proteins was similar to that of FRS2α and FRS2β since both proteins were tyrosine phosphorylated in response to FGF stimulation (Figure 2D). These results further support the notion that the three threonine-rich clusters in FRS2α are the targets of MAP kinase-dependent threonine phosphorylation.

To further confirm that the electrophoretic mobility shift of FRS2α is caused by MAPK phosphorylation, 293 cells were cotransfected with an expression vector for FRS2α together with expression vectors that direct the synthesis of chimeric proteins composed of ERK2 fused to either wild-type MEK1 or activated MEK1 (results in constitutive activation of MAPK). Lysates from these FGF-stimulated or unstimulated cells were subjected to SDS-PAGE followed by immunoblotting with anti-FRS2α antibodies. The experiment presented in Figure 2E demonstrates that the electrophoretic mobility of FRS2α was shifted in cells coexpressing activated MAPK together with FRS2α even in the absence of FGF stimulation, exhibiting a migration pattern similar to that of FRS2α immunoprecipitated from FGF-stimulated cells.

We have also analyzed the in vitro phosphorylation of FRS2α by purified activated MAPK (Figure 2F). In this experiment, FRS2α or the FRS2α-8V mutant was immunoprecipitated from lysates of unstimulated or FGF-stimulated cells and then mixed with purified activated MAPK in the presence of MgATP. The samples were subsequently subjected to SDS-PAGE followed by immunoblotting with anti-FRS2α or anti-pThr antibodies. The experiment presented in Figure 2F shows MAPK-induced threonine phosphorylation of FRS2α in vitro and a shift in the electrophoretic mobility of FRS2α similar to the shift induced by FGF stimulation. However, the FRS2α-8V mutant was not phosphorylated by MAPK or in response to FGF stimulation.

### Tyrosine Phosphorylation of FRS2α Is Enhanced by Blocking Its Threonine Phosphorylation

To further delineate the role of threonine phosphorylation of FRS2α, we performed immunoblotting analysis of anti-FRS2α immunoprecipitates with anti-p-Tyr antibodies and observed that tyrosine phosphorylation of the FRS2α-8V mutant is increased in comparison to tyrosine phosphorylation of wild-type FRS2α expressed in MEFs (Figure 3A). Moreover, enhanced tyrosine phosphorylation of the FRS2α-8V mutant could be detected even in unstimulated cells and resulted in a parallel increase in complex formation between Grb2 and FRS2α. In contrast, wild-type FRS2α was found to be constitutively phosphorylated on threonine but not on tyrosine residues in unstimulated MEFs. After FGF stimulation, FRS2α became heavily tyrosine phosphorylated and formed a complex with Grb2. In addition, upon FGF stimulation, threonine phosphorylation of FRS2α is enhanced and, as a consequence, its electrophoretic mobility is altered. Pretreatment of the cells with U0126, a MEK inhibitor, prevented threonine phosphorylation of FRS2α and consequently lengthened its electrophoretic migration on SDS-PAGE relative to that of FRS2α from cells that were not treated with the MEK inhibitor (Figure 3A). Furthermore, treatment of the cells with U0126 increased the tyrosine phosphorylation of FRS2α, which paralleled an increase in complex formation between FRS2α and Grb2 (Figure 3A). In contrast, the MEK inhibitor did not affect the electrophoretic mobility and the tyrosine phosphorylation of the FRS2α-8V mutant nor did it influence complex formation with Grb2 (Figure 3A). The bulk of the electrophoretic mobility shift is caused by threonine phosphorylation of FRS2α while tyrosine phosphorylation induces a small shift in the electrophoretic mobility of the protein. The effects of the MEK inhibitor on FRS2α and its downstream responses are specific because similar responses were not elicited by treatments with the protein kinase-C inhibitor GF109203X (Figure 3B), the PI-3 kinase inhibitor, Wortmannin (Figure 3B), or with the protein kinase-A inhibitor PKI (data not shown).

### FGF-Induced Cellular Responses Are Augmented in Cells Expressing the FRS2α-8V Mutant

We have shown that treatments that augment threonine phosphorylation of FRS2α (e.g., Okadaic acid) correspondingly decrease FGF-induced tyrosine phosphorylation of the docking protein. Conversely, treatments that attenuate threonine phosphorylation (e.g., MEK inhibitor) of FRS2α result in increased tyrosine phosphorylation of FRS2α. Similarly, FGF-induced tyrosine phosphorylation of FRS2α-8V, a mutant defective in threonine phosphorylation, is enhanced relative to tyrosine phosphorylation of wild-type FRS2α. Moreover, tyrosine phosphorylation of FRS2α-8V is detected even in unstimulated MEFs. Collectively, these experiments indicate that MAP kinase-dependent phosphorylation of FRS2α may function as a switch that negatively regulates FGF-dependent signaling pathways that are mediated by FRS2α. Indeed, the experiment presented in Figure 4A shows that the duration of tyrosine phosphorylation of the FRS2α-8V mutant is prolonged as compared to that of wild-type FRS2α expressed in the same cells. Furthermore, the interaction between FRS2α and Grb2 also follows a strikingly similar pattern (Figure 4A), suggesting that the recruitment of the guanine nucleotide exchange factor, Sos, by Grb2 is also enhanced in cells expressing the FRS2α-8V mutant. If tyrosine phosphorylation of FRS2α is enhanced by blocking its

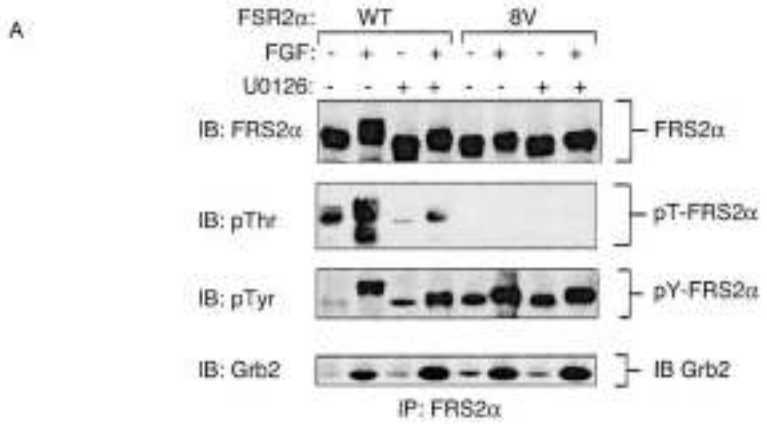
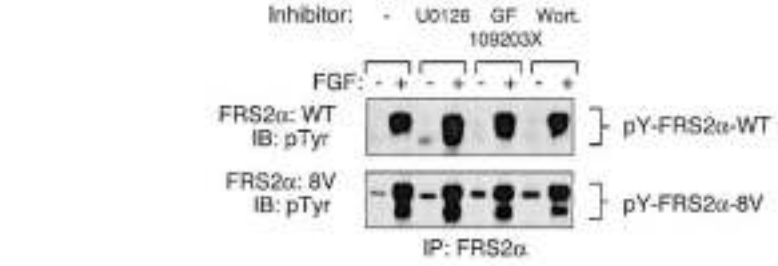



Figure 3. Inhibition of Threonine Phosphorylation and Electrophoretic Mobility Shift Enhances Tyrosine Phosphorylation of FRS2α and Complex Formation with Grb2

(A) Unstimulated or FGF-stimulated MEFs expressing FRS2α-WT or the FRS2α-8V mutant were left untreated or treated with the MEK inhibitor UO126. Cell lysates were subjected to immunoprecipitation with anti-FRS2α antibodies followed by immunoblotting with anti-FRS2α, anti-p-Thr, anti-p-Tyr, or anti-Grb2 antibodies.

(B) Unstimulated or FGF-stimulated MEFs expressing FRS2α-WT or the FRS2α-8V mutant were left untreated or treated with a MEK inhibitor (UO126), PKC inhibitor (GF109203X), or PI-3 kinase inhibitor (Wortmannin). Cell lysates were subjected to immunoprecipitation with anti-FRS2α antibodies followed by immunoblotting with anti-p-Tyr antibodies.




threonine phosphorylation, it follows that FGF-dependent signaling pathways that are dependent upon FRS2α will be potentiated in MEFs expressing the FRS2a-8V mutant. Indeed, the MAPK response is more sustained in FRS2α-8V expressing cells as compared with MAPK stimulation in cells expressing wild-type FRS2α (Figure 4B). By contrast, tyrosine phosphorylation of phospholipase Cγ was similar in FRS2α-WT and FRS2α-8V expressing cells (Figure 4C).

### Enhanced Mitogenic Response of Cells Expressing the FRS2α-8V Mutant

We next examined the biological consequences of the disruption of FRS2α threonine phosphorylation in FRS2α$^{-/-}$ MEFs expressing either wild-type or mutant FRS2α. Using the Boyden chamber assay, we observed that the mobility of unstimulated cells expressing FRS2α-8V is enhanced, and that FGF-stimulation induced stronger cell migration of MEFs expressing FRS2α-8V as compared to MEFs expressing wild-type FRS2α (Figure 5A). In addition, MEFs expressing FRS2α-8V grew faster than wild-type MEFs and exhibited anchorage-independent growth in response to FGF stimulation (Figure 5B). The transformed-like phenotype of the FRS2α-8V cells was also reflected in the ability of these cells to form colonies in soft agar (Figure 5C). This anchorage-independent growth and the ability of MEFs expressing the FRS2α-8V mutant to grow in soft agar demonstrates that the stronger mitogenic signal exerted by the FRS2α-8V mutant protein is capable of initiating certain hallmarks of cell transformation. Moreover, these experiments demonstrate that FGF-induced threonine phosphorylation of FRS2α negatively regulates tyrosine phosphorylation of the docking protein, a step crucial for recruitment and activation of multiple signal transduction pathways.

### Multiple Growth Factors Induce MAPK-Dependent Threonine Phosphorylation of FRS2α

To further understand the specificity of FRS2α threonine phosphorylation in growth factor signaling, we studied the phosphorylation profile of FRS2α in response to different growth factors. The experiment presented in Figure 6 shows that the electrophoretic mobility shift and threonine phosphorylation of FRS2α are induced in response to insulin, EGF, or PDGF stimulation. These extracellular stimuli do not induce tyrosine phosphorylation of FRS2α and, so far, there is no evidence that FRS2α plays a role in mediating their intracellular responses. The electrophoretic mobility shift of FRS2α induced by EGF stimulation was blocked by expression of a dominant interfering mutant of Ras (Ras N 17), thus confirming that kinases that act downstream of Ras are responsible for the EGF-induced response (Figure 6A). Additionally, immunobloting of anti-FRS2α immunoprecipitates with anti-p-Thr antibodies showed that EGF-induced threonine phosphorylation of FRS2α is strongly reduced by expression of Ras N-17 in PC12 cells. Similarly, the electrophoretic mobility shift of FRS2α induced by EGF, insulin, or PDGF stimulation was completely blocked by treating MEFs with the MEK inhibitor, UO126 (Figure 6B). Overall, these experiments demonstrate that

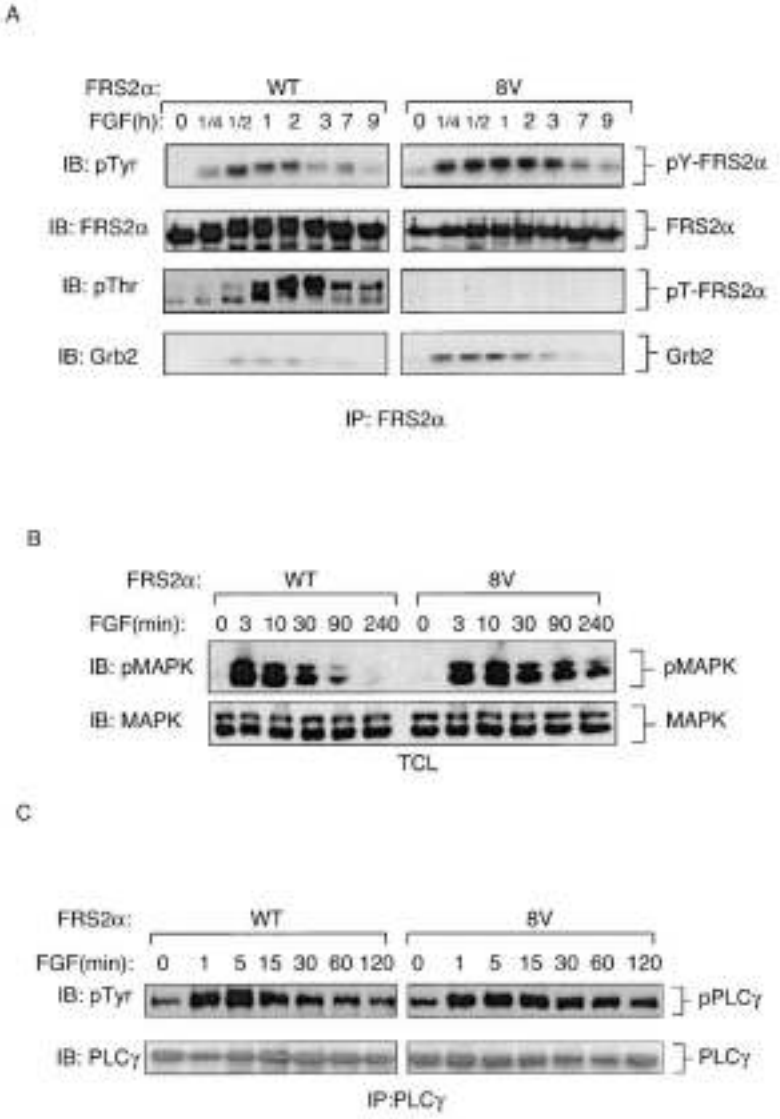



Figure 4. Negative Regulation of Tyrosine Phosphorylation of FRS2α and MAPK Stimulation by Threonine Phosphorylation of the Docking Protein

(A) The kinetics of FGF-induced tyrosine phosphorylation of FRS2α-WT or FRS2α-8V and the kinetics of FGF-induced complex formation between Grb2 and FRS2α-WT or FRS2α-8V. Cell lysates were subjected to immunoprecipitation with anti-FRS2α antibodies followed by immunoblotting with anti-p-Tyr, anti-FRS2α, or anti-Grb2 antibodies.

(B) The kinetics of FGF-induced MAP kinase stimulation in MEFs expressing FRS2α-WT or FRS2α-8V. Total cell lysates (TCL) were immunoblotted with anti-MAPK or anti-p-MAPK antibodies.

(C) The kinetics of FGF-induced tyrosine phosphorylation of phospholipase Cγ (PLCγ) in MEFs expressing FRS2α-WT or FRS2α-8V. Cell lysates were subjected to immunoprecipitation with anti-PLCγ antibodies followed by immunoblotting with anti-PLCγ or anti-p-Tyr antibodies.

MAP kinase-dependent phosphorylation of FRS2α is enhanced by stimuli that induce tyrosine phosphorylation of FRS2α (i.e., FGF) and by stimuli that do not induce tyrosine phosphorylation of the docking protein (i.e., insulin, EGF, and PDGF).

### Complex Formation between FRS2α and MAPK in FGF Stimulated Cells

We were interested in the mechanism of MAPK-dependent phosphorylation of FRS2α and how the attenuation of tyrosine phosphorylation is caused by threonine phosphorylation of the docking protein. In coimmunoprecipitation experiments, we have noticed complex formation between MAPK and FRS2α upon FGF treatment (Figure 7A). We subsequently analyzed the in vivo association between MAPK and FRS2α in FRS2α$^{-/-}$ MEFs expressing either FRS2α-WT or the FRS2α-8V mutant. In this experiment, lysates from unstimulated or FGF stimulated cells were subjected to immunoprecipitation with anti-FRS2 antibodies followed by immunoblotting with anti-MAPK antibodies, anti-p-MAPK, or anti-p-Tyr antibodies. The experiment presented in Figure 7B shows complex formation between FRS2α and the activated form of MAPK in lysates from FGF-stimulated cells, while complex formation between the FRS2α-8V mutant and MAPK was strongly reduced.

To further analyze the interaction between FRS2α and MAPK, 293 cells were cotransfected with an expression vector for FRS2α together with expression vectors that direct the expression of activated MAPK or wild-type MAPK chimeric proteins. Lysates from unstimulated or FGF-stimulated cells were subjected to immunoprecipitation with anti-MAPK antibodies followed by immunoblotting with anti-FRS2α antibodies. Only the activated form of MAPK formed a complex with FRS2α (Figure 7C). In addition, complex formation between FRS2α and MAPK was disrupted by treating the cells with the MEK inhibitor U0126 (Figure 7D). Collectively, these results show that activated MAPK forms a complex with FRS2α in response to FGF stimulation and

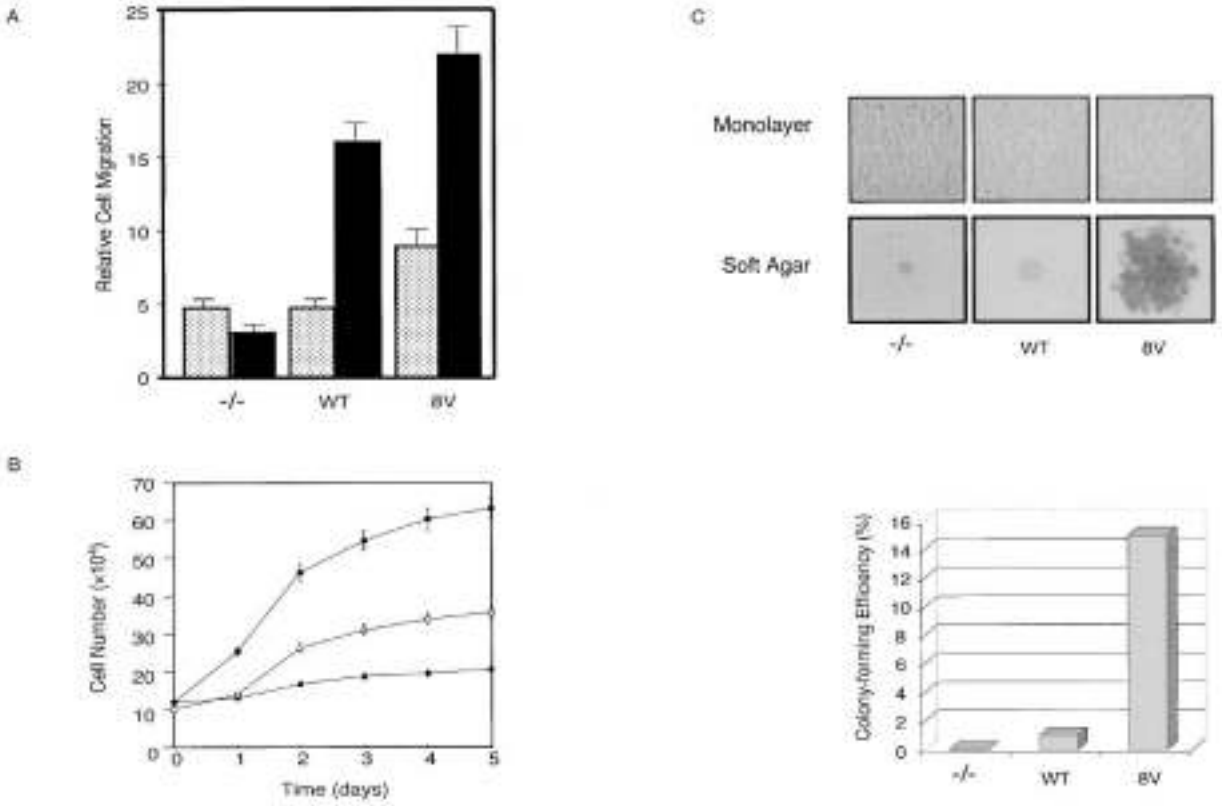



Figure 5. Enhanced Cell Motility and Colony Formation in Soft Agar of Cells Expressing the FRS2α-8V Mutant

(A) Unstimulated or FGF-stimulated MEFs expressing FRS2α-WT or FRS2α-8V were subjected to cell migration assays using the Boyden chamber. Unstimulated (open squares); FGF stimulated (closed squares) cells. The results are the average of three experiments.

(B) FGF-induced cell proliferation of MEFs from FRS2α$^{-/-}$ (closed circles), MEFs expressing FRS2α-WT (open squares), or the FRS2α-8V mutant (closed squares). Cell numbers were counted over the course of five days. The results are the average of two experiments performed in duplicates.

(C) The growth of FRS2α$^{-/-}$ MEFs and FRS2α$^{-/-}$ MEFs expressing FRS2α-WT or the FRS2α-8V mutant attached to monolayers or in 0.3% soft agar. Anchorage-independent growth of macroscopic colonies was scored after 14 days of FGF stimulation. The diagram depicts quantitation of the colony forming efficiency of the FRS2α$^{-/-}$ MEFs or the MEFs expressing FRS2α-WT or the FRS2α-8V mutant.

is likely responsible for threonine phosphorylation of FRS2α and perhaps also for the attenuation of tyrosine phosphorylation of the docking protein.

## Discussion

The docking protein FRS2α is a key mediator of signaling via FGF receptors as well as via members of the NGF and GDNF families of neurotrophic factor receptors (Kouhara et al., 1997; Hadari et al., 1998; 2001; Ong et al., 2000, 2001; Dhalluin et al., 2000; Yan et al., 2002). In this report we demonstrate that in addition to its positive regulatory roles, FRS2α functions as a key component of a MAP kinase-dependent negative feedback mechanism that ensures controlled dosage of signaling via FGF receptors (Figure 7E). Furthermore, the ability of insulin, EGF, or PDGF to induce threonine phosphorylation of FRS2α may add an additional layer of regulation to this system by providing a mechanism that ensures the balanced activation of a common signaling pathway that is stimulated by multiple receptor tyrosine kinases (Figure 7E). Since tyrosine phosphorylation is crucial for recruitment and activation of FRS2α-dependent signaling pathways, it is expected that tyrosine phosphorylation of the docking protein will be under tight control especially in a situation when the key mediator (i.e., FRS2α) is constitutively associated with the FGF receptor (Ong et al., 2000). To prevent inadvertent activation of FGF-dependent signaling pathways by random, non-ligand-dependent dimerization of FGFR molecules (Schlessinger, 2000), additional control mechanisms of key intermediates are essential. Experiments presented in this report demonstrate that FRS2α is a target of MAPK phosphorylation, activated by FGF or by other growth factors. Phosphorylation of FRS2α by MAPK occurs at eight specific threonine residues (within consensus PXTP motifs) that are clustered in three separate regions of the FRS2α molecule. Although threonine phosphorylation of FRS2α can be observed in unstimulated cells, growth factor stimulation further enhances this process. Conversely, blocking threonine phosphorylation of FRS2α results in enhanced tyrosine phosphorylation of the docking protein and a parallel increase in complex formation with Grb2 even in unstimulated cells. Consequently, FRS2α-dependent signaling pathways are more strongly activated under conditions in which threonine phosphorylation of FRS2α is prevented. Phosphorylation of all three threonine clusters appears to be

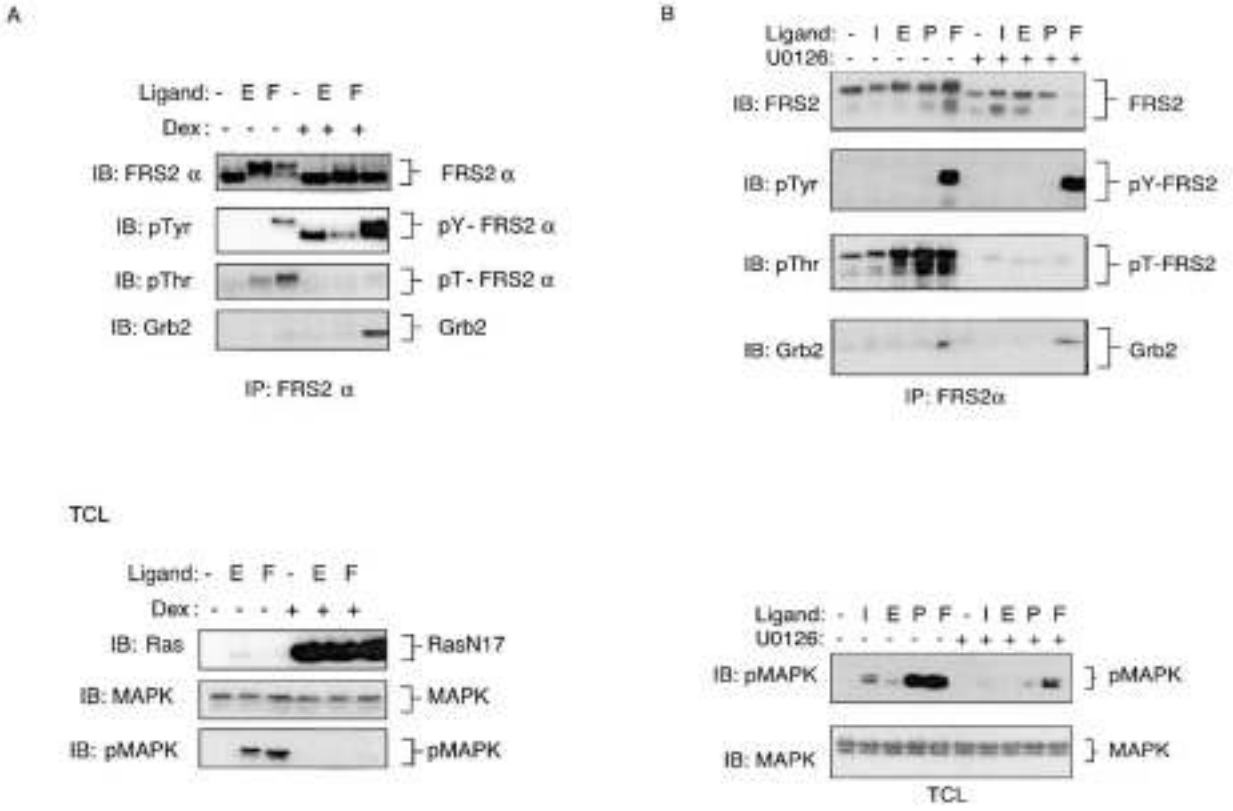



Figure 6. Multiple Growth Factors Induce Threonine Phosphorylation and Electrophoretic Mobility Shift of FRS2α

(A) PC12 cells expressing Ras N-17 in an inducible vector were treated with dexamethanone (Dex) or were left untreated. Cells were then stimulated with EGF (E) or FGF (F) followed by cell solubilization. Cell lysates were subjected to immunoprecipitation with anti-FRS2α antibodies followed by immunoblotting with anti-FRS2α, anti-p-Tyr, anti-p-Thr, or anti-Grb2 antibodies. Also shown, anti-Ras, anti-MAPK, and anti-p-MAPK immunoblots of total cell lysates (TCL).

(B) MEFs expressing FRS2α-WT were left untreated or treated with the MEK inhibitor UO126. The cells were then stimulated with insulin (I), EGF (E), PDGF (P), or FGF (F), followed by cell solubilization. Cell lysates were subjected to immunoprecipitation with anti-FRS2α antibodies followed by immunoblotting with anti-FRS2α, anti-p-Tyr, anti-p-Thr, or anti-Grb2 antibodies. Also shown, an anti-MAPK and anti-p-MAPK immunoblots of total cell lysates (TCL).

necessary for preventing inadvertent tyrosine phosphorylation of FRS2α in quiescent cells. Thus, in FGF stimulated cells, threonine phosphorylation of FRS2α functions as part of a negative feedback mechanism.

The second member of the FRS2 family of docking proteins, FRS2β, is also tyrosine phosphorylated in response to FGF stimulation. However, unlike FRS2α, the closely related FRS2β protein lacks the putative MAPK consensus phosphorylation sites; it is not phosphorylated on threonine residues and does not undergo an electrophoretic mobility shift in response to FGF stimulation. The lack of MAPK phosphorylation sites on FRS2β may indicate that FRS2β is subjected to different regulatory processes as compared to FRS2α. While the neuronally restricted expression pattern of FRS2β may suggest its involvement in signaling by neurotrophic factors, FRS2α, on the other hand, exhibits a broader expression pattern (including expression in the nervous system) highlighting its more pleotropic involvement in growth factor signaling. In addition, analysis of FRS2α$^{-/-}$ mice demonstrated that FRS2α plays a critical role in embryonic development as a key mediator of FGF receptor signaling. FRS2α is likely to function as a key element in the control of signaling by both FGF and neurotrophic factors in multiple cells, tissues, and organs. The close association of FRS2α with inactive FGF receptors may require stringent control mechanisms for regulation of tyrosine phosphorylation of FRS2α in unstimulated cells. Growth factor induced threonine phosphorylation of FRS2α by MAPK may provide a molecular switch that adjusts the degree to which FRS2α is tyrosine phosphorylated and associated with downstream effector proteins. The interaction between FRS2β and receptors for neurotrophic factors involves a more conventional mechanism. FRS2β binds only to activated TrkA; complex formation is mediated by binding of the PTB domain to a canonical tyrosine phosphorylated NPXY motif in the juxtamembrane domain of TrkA. Complex formation between FRS2β and TrkA followed by subsequent tyrosine phosphorylation of FRS2β are controlled by activation and autophosphorylation of TrkA. This well-controlled process may not require an additional level of regulation by MAP kinase phosphorylation to control the level of tyrosine phosphorylation of FRS2β in unstimulated cells, and as such, FRS2β may have evolved without the need to become threonine phosphorylated by MAPK.

An important question is how threonine phosphorylation

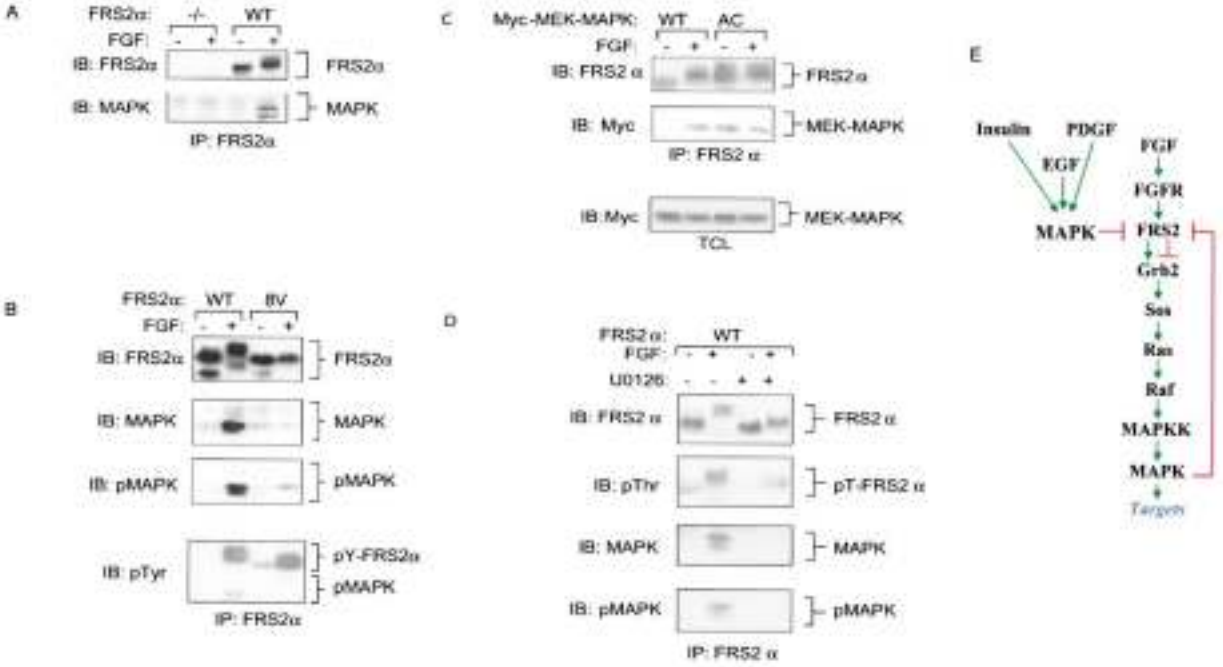



Figure 7. Complex Formation between FRS2α and Activated MAPK in FGF-Stimulated Cells

(A) Unstimulated or FGF-stimulated FRS2α$^{-/-}$ MEFs or MEFs expressing FRS2α-WT were subjected to immunoprecipitation with anti-FRS2α antibodies followed by immunoblotting with anti-FRS2α or anti-MAPK antibodies

(B) Unstimulated or FGF-stimulated MEFs expressing FRS2α-WT or FRS2α-8V were subjected to immunoprecipitation with anti-FRS2α antibodies followed by immunoblotting with anti-FRS2α, anti-MAPK, anti-p-MAPK, or anti-p-Tyr antibodies.

(C) HEK 293 cells cotransfected with expression vectors for FRS2α together with expression vectors for either wild-type Myc-tagged-MEK-MAPK (WT) or activated Myc-tagged-MEK-MAPK (AC). Lysates from unstimulated or FGF-stimulated cells were subjected to immunoprecipitation with anti-FRS2α antibodies followed by immunoblotting with anti-FRS2α or anti-Myc-tag antibodies. Also shown, anti-Myc-tag immunoblots of total cell lysates (TCL).

(D) Unstimulated or FGF-stimulated MEFs expressing FRS2α-WT were left untreated or treated with the MEK inhibitor UO126. Cell lysates were subjected to immunoblotting with anti-FRS2α, anti-p-Thr, anti-MAPK, or anti-p-MAPK antibodies.

(E) A scheme depicting MAPK-mediated phosphorylation and attenuation of signaling via the docking protein FRS2α. FGF-induced activation of FGFR leads to tyrosine phosphorylation of FRS2α followed by Grb2/Sos-mediated activation of Ras. Activated Ras stimulates a kinase cascade composed of Raf, MAPKK (MEK), and MAPK (ERK). MAPK activated by FGF receptor or in response to insulin, PDGF, or EGF stimulation phosphorylates FRS2α on at least 8 threonine residues resulting in reduced tyrosine phosphorylation of FRS2α, diminished recruitment of Grb2, and other effector proteins that function downstream of FRS2α.

negatively regulates FGFR-mediated tyrosine phosphorylation of the docking protein. Does threonine phosphorylation make FRS2α a poorer substrate for activation (tyrosine phosphorylation), or a better substrate for inactivation (dephosphorylation)? One possible explanation is that complex formation between FGFR and the threonine phosphorylated form of FRS2α interferes with the intrinsic catalytic activity of the FGFR protein tyrosine kinase (PTK) domain. Alternatively, threonine phosphorylation may interfere with complex formation between FRS2α and FGFR, resulting in decreased tyrosine phosphorylation of the docking protein. However, we were unable to detect any change in autophosphorylation of FGFR, tyrosine phosphorylation of Shc (data not shown), or phospholipase C-γ, two well-known substrates of FGFR, in MEFs expressing either wild-type or the FRS2α-8V mutant protein, thus ruling out the possibility that the intrinsic PTK activity of FGFR is negatively regulated by threonine phosphorylation of FRS2α. Furthermore, we were also unable to detect changes in complex formation between FGFR with either FRS2α-WT or the FRS2α-8V mutant protein, indicating that the PTB-mediated complex formation with FGFR is not influenced by threonine phosphorylation of the docking protein.

We have demonstrated in this report that in response to FGF stimulation, the activated form of MAPK forms a complex with FRS2α. Although the molecular details of the mode of interaction between activated MAPK and FRS2α remain to be determined, the complex formed between activated MAPK and FRS2α may interfere with the capacity of FGFR to interact with and tyrosine phosphorylate FRS2α. This mechanism resembles the direct interaction between the mating-specific MAPK and the pheromone-responsive G protein α (Gα) of yeast resulting in downregulation of the pheromone-induced Gα-MAPK pathway (Metodiev et al., 2002). An additional non-mutually exclusive possibility is that threonine phosphorylation induces a structural change in FRS2α rendering the docking protein a poorer substrate toward the FGFR. It should be noted that several p-Thr sites lie in close proximity to the p-Tyr sites in the primary structure of FRS2α. Threonine phosphorylation may confer a local change in the structure of the docking protein proximal to the tyrosine phosphorylation sites.

This together with the presence of bound MAPK protein in close proximity may interfere with FGFR-mediated tyrosine phosphorylation of FRS2α. An additional non-mutually exclusive mechanism is that threonine phosphorylation will trigger binding of protein(s) that bind to their targets in a phosphorylation-dependent manner by means of their 14-3-3, WW, or FHA domains (Yaffe and Elia, 2001; Sudol, 1998; Tzivion et al., 2001; Tzivion and Avruch, 2000). Phospho-threonine-mediated complex formation with other protein(s) may interfere with the interaction between the catalytic domain of FGFR and FRS2α, a step necessary for tyrosine phosphorylation to take place. Furthermore, if the bound protein is endowed with intrinsic protein tyrosine phosphatase activity, complex formation may reduce tyrosine phosphorylation of FRS2α by a phosphothreonine-dependent phosphotyrosine dephosphorylation.

Phosphorylation on Ser/Thr residues is a well-known mechanism for the regulation of activity of proteins involved in growth factor receptor signaling. It has been shown that phosphorylation of the EGF receptor on Thr654 by PKC leads to modulation of the EGF receptor ligand binding activity and changes in intracellular trafficking of EGF receptor following endocytosis (Cochet et al., 1984; Livneh et al., 1988). In addition, phosphorylation of IRS1 (insulin receptor substrate 1) by PKC, Erk, and Akt results in reduced complex formation with PI-3 kinase (De Fea et al., 1997a, 1997b; Li et al., 1999). It was also shown that hyperphosphorylation of Gab1 on Ser/Thr residues negatively regulates hepatocyte growth factor-mediated biological responses (Gual et al., 2001). However, another study reported that phosphorylation of Gab1 by MAP kinase enhances complex formation with PI-3 kinase resulting in stimulation of PI-3 kinase activity (Yu et al., 2001). Another example of a negative regulatory role of MAP kinase is illustrated by the phosphorylation of Smad1 to prevent nuclear accumulation and transcriptional activity in response to BMP stimulation (Kretzschmar et al., 1997).

In summary, experiments presented in this report demonstrate that the same molecule responsible for the recruitment of positive regulators can utilize elements of the same pathway for a negative feedback mechanism resulting in signal attenuation. MAPK response induced by FGF and other growth factors controls multiple intracellular responses as well as the tuning of its own activity by negatively regulating a key element required for its own activation.

## Experimental Procedures

### Cell Lines

Mouse embryonic fibroblasts (MEFs) and HEK 293 cells were cultured in the presence of DMEM containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, and penicillin/streptomycin. PC12 cells were grown in DMEM-FBS supplemented with 10% heat-inactivated horse serum (GIBCO/BRL). Generations of FRS2α$^{-/-}$ cells expressing the different FRS2α mutant were previously described (Hadari et al., 2001). Prior to growth factor stimulation, cells were starved in serum-free medium overnight. PC12 cells expressing a conditional Ras-N17 mutant were treated with Dexamethasone (Dex) as previously described (Kouhara et al., 1997).

### Growth Factors, Inhibitors, Antibodies, and Plasmids

EGF, PDGF (Invitrogen), and insulin (Sigma) were used at the following concentrations: EGF, 100 ng/ml; PDGF, 25 ng/ml. Insulin: 100 ng/ml. FGF1 (Spivak-Kroizman et al., 1994; 100 ng/ml) and heparin (Sigma, 15 μg/ml) were added together for stimulation of FGF receptors. Protein kinase inhibitors were used at the following concentrations: MEK (UO126, Promega, 10 mM); PKC (GF109203X, Biomol, 3 mM); PKA (PKI, Biomol, 3 mM); and PI-3K (Wortmannin, Sigma, 200 nM), Okadaic acid (Sigma, 20 nM), alkaline phosphatase (Sigma, 5 μg/sample), and Dexamethasone (Sigma, 2 μM). Antibodies against FRS2α, Grb2, p-Tyr, and Ras were previously described (Kouhara et al., 1997). Antibodies again Myc-tag, MAPK, Phospholipase Cγ (PLCγ), and anti-mouse HRP were purchased from Santa Cruz Biotechnologies. Antibodies directed against the p-Thr phosphorylation site of MAPK (PXpTP) were obtained from Cell Signaling. Anti-FLAG antibodies (M2) were obtained from Sigma. All antibodies were dissolved in 1XTBS/5% BSA for immunoblotting experiments. The viral expression plasmids were constructed in pBABE/puro as described (Hadari et al., 2001). All plasmids used in transient expression experiments were constructed in pRK5 (Kouhara et al., 1997). The Myc-tag MEK-MAPK plasmids were obtained from Melanie Cobb. Purified activated ERK2 expressed as a GST fusion protein was purchased from Upstate Biotechnology (UBI). The in vitro kinase assay was performed according to the manufacturer's protocol using MAP kinase assay kit (UBI).

### Immunoprecipitation and Immunoblotting Analysis

Cells were lysed in 20 mM Hepes, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 5% Glycerol, 10 mM pyrophosphate, 1 mM $NaVO_3$, 1 mM PMSF, 5 μg/ml aprotinin, and 5 μg/ml leupeptin (pH 7.5). The procedures applied for cell solubilization immunoprecipitation and immunoblotting with the different antibodies were previously described (Batzer et al., 1994; Mohammadi et al., 1996).

### Cell Migration, Cell Proliferation, and Colony Formation in Soft Agar

Cell migration assays were performed using Boyden chambers (Costar). In brief, chambers were precoated with 10 mg/ml collagen for 1 hr at 37°C. Chemoattractants in 1.5 ml PBS or control solution were added to the bottom chamber. MEFs were starved overnight, harvested with buffer containing 25 mM HEPES and 5 mM EDTA (pH 7.5), pelleted, and then resuspended to $5 \times 10^5$ cells/ml in PBS. Cell suspension (0.5 ml) was placed on the top membrane, and chambers were incubated for 4 hr at 37°C. Cells were then removed from the top part of the membrane and the cells attached to the bottom membrane were fixed with methanol, stained with hematoxylin, and counted (Hadari et al., 2001). Cell proliferation assays were performed according to published procedure (Spivak-Kroizman et al., 1994). For soft agar colony assays, cells were plated on a layer of 0.75% soft agarose at different cell densities ($10^3$, $10^5$, $10^4$) in 0.3% soft agarose. Anchorage-independent growth of macroscopic colonies was scored after 14 days.

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