Direct Cell Cycle Regulation by the Fibroblast Growth Factor Receptor (FGFR) Kinase through Phosphorylation-dependent Release of Cks1 from FGFR Substrate 2

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Fibroblast growth factors (FGFs) are upstream activators of the mitogen-activated protein kinase pathway and mitogens in a wide variety of cells. However, whether the mitogen-activated protein kinase pathway solely accounts for the induction of cell cycle or anti-apoptotic activity of the FGF receptor (FGFR) tyrosine kinase is not clear. Here we report that cell cycle inducer Cks1, which triggers ubiquitination and degradation of p27Kip1, associates with the unphosphorylated form of FGFR substrate 2 (FRS2), an adaptor protein that is phosphorylated by FGFR kinases and recruits downstream signaling molecules. FGF-dependent activation of FGFR tyrosine kinases induces FRS2 phosphorylation, causes release of Cks1 from FRS2, and promotes degradation of p27Kip1 in 3T3 cells. Since degradation of p27Kip1 is a key regulatory step in activation of the cyclin E/Cdk complex during the G1/S transition of the cell cycle, the results suggest a novel mitogenic pathway whereby FGF and other growth factors that activate FRS2 directly activate cyclin-dependent kinases.

The fibroblast growth factor (FGF) family is a ubiquitously expressed transmembrane signaling family that elicits receptor-mediated regulatory effects on cell growth, function, differentiation, and death. The FGF ligands are single polypeptides consisting of 22 genetically distinct homologues, and the FGF receptors (FGFRs) are transmembrane tyrosine kinases encoded by four homologous gene products, which complex with pericellular matrix heparan sulfates independent of the FGF ligand (1, 2). Binding of FGF ligands to FGFR-heparan sulfate complexes activates the kinase activity and transmits regulatory signals to downstream signaling mediators or targets. Accumulating reports demonstrate that FGFRs elicit diverse regulatory activities that are cell type- and FGFR-specific as well as redundant among the four FGFRs. The molecular mechanisms that would explain both specificity and redundant activities are not well understood. Two proximal substrates (FRS1 and FRS2) interact with the activated FGFR tyrosine kinases that are tyrosine-autophosphorylated. FRS1 is phospholipase Cγ, which binds to phosphorylated tyrosine 766 of FGFR1, and is phosphorylated by the kinase. Activation of FRS1 is not obligatory for the mitogenic activity of FGFR1 (3, 4). However, it is important for prostate epithelial cells to acquire a mitogenic response to FGFR1 and for other cellular activities stimulated by phospholipase Cγ (5).

FRS2 (also called SNT1, for suc1-associating nuclear target protein) is an adaptor protein that is anchored to the plasma membrane by virtue of myristoylation at the N terminus (6–8). FRS2 interacts with the intracellular juxtamembrane sequence of the FGFR through a conserved phosphotyrosine binding (PTB) domain adjacent to the C terminus of the myristoylation consensus sequence (9, 10). The PTB domain of FRS2 is homologous to and exhibits a similar three-dimensional structure as PTB domains of several other signaling adaptor proteins, including insulin receptor substrate 1. Phosphorylation of FRS2 in response to FGF treatment is associated with the FGF mitogenic signaling (6). The C-terminal half of FRS2 has multiple candidate phosphorylation sites as well as numerous signaling molecule binding sites, which include Grb2 that recruits upstream activators of the extracellular signal-regulated kinase 1/2 in the MAP kinase pathway. Therefore, FRS2 is considered an adaptor that connects FGFR to the MAP kinase pathway (5, 11–15). The fact that not all FGF signaling is tightly associated with activation of the MAP kinase pathway (16, 17) prompted us to investigate alternative and potentially more direct means by which the FGFR transmits mitogenic signals.

The interaction of FRS2 with p13suc1, a yeast cyclin-dependent kinase (Cdk)-binding protein important for cell cycle regulation (18), has been utilized together with anti-phosphotyrosine antibodies to precipitate and assess FGF-dependent phosphorylation of FRS2 for decades. Although a useful experimental tool, the interaction has not been associated with FGF signal transduction. Two mammalian homologues of Suc1, Cks1 and Cks2, have been identified (19–21). Cks1 is required for p27Kip1 to interact with the Skp-2 (S-phase kinase-associated protein 2) ubiquitin ligase complex and promotes p27Kip1 degradation (22–24). The degradation of p27Kip1 in the late G1 phase activates Cdk2-cyclin E and Cdk2-cyclin A complexes that, in turn, signal the cells to enter the S phase of the cell cycle (22, 23).

Despite the fact that Cks1 and Cks2 are homologues, the function of Cks2 is not clear. Cks2 has been implicated in the first metaphase/anaphase transition of mammalian meiosis (25). Mice lacking Cks2 are viable, but both sexes are sterile, in

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1 The abbreviations used are: FGF, fibroblast growth factor; FGFR, FGF receptor; FRS, FGF receptor substrate; GST, glutathione S-transferase; PTB, phosphotyrosine binding; MAP, mitogen-activated protein; Cdk, cyclin-dependent kinase; GFP, green fluorescent protein.
contrast to mice lacking Cks1 that are smaller than wild type (23). Cells derived from the Cks1 null mice proliferate at a reduced rate, consistent with elevated levels of p27<sup>Kip1</sup> (23). Cellular levels of p27<sup>Kip1</sup> are tightly controlled. The expression of p27<sup>Kip1</sup> is frequently down-regulated in human tumors (26, 27), and the reduction correlates with a poor prognosis (28).

Although the interaction of Cks1 with p27<sup>Kip1</sup> and Skp-2 triggers the G<sub>1</sub>/S transition by inducing p27<sup>Kip1</sup> ubiquitination and degradation, the potential upstream regulators of Cks1 have not been identified. Because of the homology of Suc1 to Cks1 and the historic interaction of Suc1 with the major FGFR kinase substrate FRS2, we asked whether the FGFR kinase might directly affect Cks1-mediated p27<sup>Kip1</sup> degradation as a mechanism of FGFR induction of the G<sub>1</sub>/S transition. Here we report that Cks1 associated with unphosphorylated FRS2 through the sequence encoding the PTB and myristoylation domains. Phosphorylation of FRS2 by the FGFR1 kinase in response to activating FGF ligands reduced the interaction and induced p27<sup>Kip1</sup> degradation in mouse 3T3 cells. The release of Cks1 from FRS2 via activation of the FGFR kinase suggests a novel signaling pathway whereby a transmembrane receptor tyrosine kinase may directly activate the cell cycle through Cks1-mediated activation of cyclin-dependent kinases.

**EXPERIMENTAL PROCEDURES**

**Construction and Expression of Recombinant Proteins**

Construction of Cks1-GST/FGFR Fusion Proteins—Full-length cDNA for mouse Cks1 was amplified by reverse transcription-PCR from mouse embryonic RNA pools with forward primer cks1a (CTCGTCGAAGCGGATCATGTCGGACACAACA) and reverse primer cks1b (CTG-CAGGTACCTTCTTTGTGTTGT). Similarly, full-length cDNA for Cks2 was amplified by reverse transcription-PCR with forward primer cks2a (TTTCTGAGGGCGGCGACGAGTAGGCCAC) and reverse primer cks2b (TAAGGTACCTTTCTTTGTGTTGT). The PCR products were cloned in pBluescript SK vector at PstI and KpnI restriction sites. After sequence verification, the PCR fragments of Cks1 and Cks2 were in-frame ligated to the 5′-end of the coding sequence for *Schistosoma japonicum* glutathione S-transferase (GST). The resulting cDNAs were subcloned into vector pVL1393 for preparation of recombinant baculoviruses. Expression of Cks1- and Cks2-GST fusion proteins was evaluated with the anti-GST polyclonal antibody (29). The Cks cDNAs were also amplified by PCR with primer FRS2Fs for expression in insect cells as GST fusion proteins that had a green fluorescent protein (GFP) tag at the N terminus or into vector pEGFP-C3 for expression as Cks-GFP fusion proteins that had a GFP tag at the C terminus.

**Construction of FRS2 Mutants**—The cDNA encoding the N terminus of ΔmFRS2-His that had a His tag at the C terminus was PCR-amplified from the full-length FRS2 coding sequence with forward primer dm1 (CTGTTGGCAGGCTTGAGATATAGACAGT) and reverse primer dm2 (TCCACTAATCTGTCTCAGAC). The cDNA coding for ΔpFRS2-His was PCR-amplified from the same template with forward primer ps1 (ACACCTGGGCGATGATTTGGCTCTGACAG) and reverse primer FRS2shib (5). The cDNA coding for PTB-His was PCR-amplified with forward primer FRS2a (5) and reverse primer ptb2 (GTTCGAGACTTACAATCTGGAGT); the cDNA coding for ΔmPTB-His was PCR-amplified with forward primer dm1 and reverse primer ptb2. The cDNA fragments were ligated to C-terminal coding sequences of FRS2-His, and the resulting fragments were cloned in vector pBluescript SK for sequence verification. Due to the lack of a translational initiation site, coding sequences for ΔmFRS2 and ΔmPTB were in-frame ligated to pCMV-Tag2 vector containing an artificial FLAG tag downstream of the translational initiation site. The molecular weights of the fusions were slightly higher than those of FRS2 and PTB, respectively, due to coding sequences of the vector. The cDNA fragments were then subcloned to vector pVL1393 for preparation of recombinant baculoviruses (29). Full-length FRS2-His fusion proteins were prepared as previously described (30).

**Affinity Pull-down Assays**

**Pull-down with Glutathione Beads**— sf9 cells (5 × 10<sup>6</sup>) were infected with the indicated recombinant viruses for 3 days and lysed with 0.5% Triton X-100-phosphate-buffered saline. The cell lysates were mixed as indicated and incubated with glutathione beads (Amersham Biosciences) at 4 °C for 2 h. The beads were washed with 0.1% Triton-phosphate-buffered saline four times, and specifically bound fractions were recovered with the SDS-sample buffer.

**Pull-down with Nickel Beads**—The cell lysates were mixed as indicated and diluted with equal volumes of the washing buffer (0.5 M NaCl, 0.1 M imidazole, and 10 mM Tris-HCl, pH 7.5). The mixtures were gently rocked with 30 μl of nickel-agarose beads (Amersham Biosciences) at 4 °C for 1 h. The FRS2-His-nickel-agarose complexes were then washed three times with the washing buffer, and specifically bound fractions were recovered with the SDS-sample buffer.

**Phosphorylation of FRS2 by FGFR1 Kinase**— sf9 insect cells (5 × 10<sup>6</sup>) were co-infected with the indicated FRS2-His and FGFR1-bearing baculoviruses for 3 days. The cells were incubated with 25 μg/ml heparin and 20 ng/ml FGFR1 for 30 min at 27 °C where indicated and lysed as described above. The cell lysates were mixed and incubated with glutathione beads or nickel beads as indicated. Specifically bound fractions were divided into three aliquots, and electrophoresed to nylon membranes. Tyrosine-phosphorylated FRS2 and FGFR1 were detected with anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology). The total FRS2-His proteins were detected with anti-His tag antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and total FGR1 proteins were detected with anti-FGFR1 antibody 17A3 as indicated. The corresponding bands were visualized with ECL-plus chemiluminescent detection reagents (Amersham Biosciences).

**Quantitation of Cellular p27<sup>Kip1</sup> Proteins**—The cells (2 × 10<sup>6</sup>) in 6-well plates were brought to quiescence by incubation in medium containing 0.1% fetal bovine serum for 48 h. Then the cells were incubated with cycloheximide (2 mg/ml) for 1 h where indicated, followed by incubation with FGF1 (20 ng/ml) and heparin (2 μg/ml) for the indicated time period. The cells were then lysed with 0.5% Triton-phosphate-buffered saline. Protein concentrations of the lysates were determined using the BCA protein assay reagent (Pierce). Aliquots equal to 5 μg of proteins were subjected to SDS-PAGE and then electrophoresed to nylon membranes. Cellular levels of p27<sup>Kip1</sup> were detected with anti-p27<sup>Kip1</sup> antibody (Santa Cruz Biotechnology). The corresponding bands were visualized with the ECL-plus chemiluminescent detection reagents.

**RESULTS**

**FRS2-His Was Tyrosine-phosphorylated by the FGFR1 Kinase in sf9 Cells**—To generate enough recombinant FRS2 proteins for biochemical studies, recombinant baculovirus bearing coding sequence for FRS2-His with a hexahistidine tag at the C terminus was generated. To determine whether FRS2-His was phosphorylated by the FGFR1 kinase, FRS2-His was co-expressed with FGFR1 kinase in sf9 cells and pulled down with nickel-Sepharose beads. The bound fractions were subjected to Western blot with anti-phosphotyrosine antibody 4G10 (Fig. 1A). Surprisingly, FRS2-His was specifically tyrosine-phosphorylated by the FGFR1 kinase. Immunoblotting with anti-phosphotyrosine antibody revealed multiple bands with apparent molecular masses ranging from 55 to 85 kDa (Fig. 1A), which reflects differential phosphorylation of FRS2 (5, 31). To determine whether the phosphorylated FRS2-His exhibited the expected association with p13<sup>mut</sup>, the recombinant FRS2-His was incubated with p13<sup>mut</sup>-agarose beads, and the bound fractions were subjected to Western blotting with the indicated antibodies. Surprisingly, FRS2-His protein was pulled down with the p13<sup>mut</sup>-GST beads independent of the presence of the FGFR1 kinase (Fig. 1B). This showed that the interaction of FRS2 and p13<sup>mut</sup> was tyrosine phosphorylation-independent.

**Phosphorylation-dependent Association of FRS2 with Cks1**—To determine whether the mammalian counterparts of yeast p13<sup>mut</sup> also associated with FRS2 and played roles in FGF signaling, mouse cDNAs coding for Cks1 and Cks2 were cloned and expressed with a GST tag at the N terminus to facilitate purification and identification (32). The Cks1-GST and Cks2-GST fusion proteins expressed in sf9 cells were soluble and bound to GSH-agarose beads (Fig. 2). In addition, Cks1-GFP and Cks2-GFP that had a GFP tag either at the N terminus or the C terminus were distributed in both cytosol and nucleus when overexpressed in NIH 3T3 cells (Supplemental Fig. 1).
This is similar to p13suc1 that gradually accumulates in the nucleus after it is microinjected into the cells (33).

To determine whether FRS2 associated with Cks1 and Cks2 and whether the association was tyrosine phosphorylation-dependent, s9 cell-expressed FRS2 recombinant proteins, with and without coexpression with the FGFR1 kinase, were applied to GSH-agarose beads together with Cks1-GST or Cks2-GST. The bound fractions were divided into three aliquots and Western blotted with the indicated antibodies (Fig. 2A). Co-expression with the FGFR1 kinase abrogated the FRS2-Cks1 interaction, whereas it only reduced the FRS2-Cks2 interaction (Fig. 2A, top panel). Consistently, Western blot with anti-phosphotyrosine antibody revealed that more phosphorylated FRS2 was pulled down by Cks2 than by Cks1 (Fig. 2A, middle panel). Under similar conditions, FRS2 was not pulled down by GSH-agarose beads in the presence or absence of the FGFR1 kinase (Fig. 2B). This indicates that the pull-down occurs through interactions between FRS2 and Cks1-GST or Cks2-GST.

To confirm that the negative impact of FGFR1 kinase on FRS2-Cks1 interactions was due to activity of FGFR1 kinase and not due to competition between Cks1 and FGFR in interaction with FRS2, similar experiments were carried out with FRS2 coexpressed with a kinase-inactive mutant of FGFR1 (R1KN). The results showed that R1KN failed to phosphorylate FRS2 and also failed to abrogate the FRS2-Cks1 interaction (Fig. 2A). Together with the fact that the direct binding of FRS2 and FGFR1 is weak, since most of FRS2 cannot be pulled down by FGFR1, the data suggest that there is no competition between Cks and FGFR1 for interaction with FRS2. To determine whether the incubation with Cks1-GST or Cks2-GST caused FRS2 degradation, FRS2-His, with and without coexpression with FGFR1 kinase, was incubated with lysates of s9 cells expressing Cks1-GST or Cks2-GST at 4 °C for 2 h. The FRS2-His in the mixtures was then pulled down with nickel-agarose beads, and the bound fractions were analyzed with the antibodies indicated in Fig. 3. The results showed that incubation with Cks1- and Cks2-GST did not induce FRS2 degradation. Together the results here suggest that unphosphorylated FRS2 associates with both Cks1 and Cks2 and that tyrosine phos-
through the ubiquitination pathway during the G1/S transition of the cell cycle and promotes cell cycle progression (22, 23). To determine whether activation of the FGFR1 kinase induced degradation of p27\(^{kip1}\) through releasing Cks1 from FRS2, NIH 3T3 cells expressing endogenous FGFR1 kinase (5) were synchronized by incubation in medium containing 0.1% serum for 48 h and then exposed to FGF2 for up to 48 h. The cellular levels of p27\(^{kip1}\) protein were measured with anti-p27\(^{kip1}\) antibodies (Fig. 6A). The results demonstrated that FGF2 significantly induced reduction of p27\(^{kip1}\) at the protein level 6 h after the treatment.

Cells constantly synthesize p27\(^{kip1}\) during the cell cycle and promotes cell cycle progression (22, 23). To determine whether activation of the FGFR1 kinase accelerated p27\(^{kip1}\) degradation instead of reducing new protein synthesis, quiescent 3T3 cells were treated with cycloheximide, a protein synthesis inhibitor, for 1 h before incubation with FGF2. Fig. 6B shows that p27\(^{kip1}\) protein in the cycloheximide-treated cells was reduced 1 h after incubation with FGF2 and then decreased to below the detection limits 6 h after incubation with FGF2. This was significantly faster than that of the cells without exposure to FGF2 or cycloheximide. The results revealed that activation of the FGFR1 kinase accelerates degradation of p27\(^{kip1}\), and the degradation is independent of new protein synthesis. This suggests that degradation of p27\(^{kip1}\) is a result of cascade reactions in the existing signaling networks (Fig. 7) rather than a result of changes at the gene expression level.

**DISCUSSION**

The interaction between p13\(^{suc1}\) and FRS2 has been utilized as an experimental tool to assess FGFR-induced phosphorylation of FRS2 for decades. However, the interaction has largely been assumed to be fortuitous and not of functional significance in FGF signaling. Here we report that Cks1, a mammalian homologue of p13\(^{suc1}\), associated with the non-tyrosine-phosphorylated FRS2. Activation of the FGFR1 tyrosine kinase led to tyrosine phosphorylation of FRS2, released Cks1 from FRS2, and promoted p27\(^{kip1}\) degradation. In addition, FRS2 bound to Cks1 via the conserved N-terminal sequence consisting of the PTB and myristoylation domains; deletion of the sequence abrogated the association. The FRS2 myristoylation site is composed of 6 amino acid residues (MGSCCS) located at the N terminus, which affixes FRS2 to the plasma membrane when it is myristoylated. It is likely that myristoylation and the membrane anchorage of FRS2 are important for the three-dimensional structure of the PTB domain to interact with Cks1 and Cks2, although the possibility that the myristoylation domain is a part of the Cks binding site cannot be ruled out.

FRS2 utilizes different sets of amino acid residues for interacting with Trk and other receptor tyrosine kinases (9, 10). Currently, it is unknown how the PTB domain interacts with Cks1, although the data here suggest that the sequence downstream of the PTB domain inhibits the interaction when it is phosphorylated by FGFR1 kinase. Unlike Cks1 that only bound to unphosphorylated FRS2, Cks2 also bound to phosphorylated FRS2. The functional significance of the interaction remains to be determined.

p27\(^{kip1}\) belongs to the Cip/Kip family that negatively regulates Cdk kinase activity and plays a pivotal role in regulating mitogenic responses of cells to extracellular signals. The mRNA level of p27\(^{kip1}\) is fairly constant during cell cycles, and the cellular level of p27\(^{kip1}\) proteins is regulated primarily at the posttranslational level through ubiquitination-mediated proteolysis. Cks1 is required for association of p27\(^{kip1}\) to the SCF ubiquitin ligase complex consisting of S-phase-associated protein 2 (Skp2), Cul-1, and ROC1/Rbx1. The results here show
that unphosphorylated FRS2 bound to Cks1; the FGFR phosphorylates FRS2 upon stimulation by the FGF and releases Cks1 from FRS2. Thereafter, the freed Cks1 may induce degradation of p27Kip1 and signal the cells entering mitosis by activating Cdk-cyclin complexes (22, 23). The results firstly suggest that the FGFR1 tyrosine kinase may transmit mitogenic signals to downstream cascades directly through regulating Cdk-cyclin complexes, as illustrated in Fig. 7.

Expression of p27Kip1 is frequently down-regulated at the protein and mRNA levels in tumor cells (28), and the decrease is significantly correlated with malignancy of tumors (35, 36). It has been proposed that p27Kip1 acts as a rheostat rather than...
as an on-off switch to control growth and neoplasia, since tumor suppression by p27Kip1 is critically dependent on the absolute level of p27Kip1 protein within cells (28). Unlike epithelial cells from benign prostate tumors and normal prostate that express the FGFR2 isoform, malignant prostate cells often express the nonresident FGFR1 isoform (1, 5, 37–39). Ectopic appearance of constitutively active FGFR1 kinase in prostate epithelial cells induces prostate hyperplasia and high grade prostatic intraepithelial neoplasia in transgenic mice (40, 41). Therefore, chronic FGFR1-mediated p27Kip1 degradation may play a role in perturbation of prostate tissue homeostasis and tumor progression. Sequestration of Cks1 by FRS2 and its control by FGFR1 kinase may be of therapeutic value in cancer prevention and treatment.

The kinase domains of the four FGFRs share a high similarity in amino acid sequence and elicit similar and redundant regulatory activities in some experimental systems (1). In other experimental systems, the FGFRs elicit individual isotype-specific and sometimes even opposite regulatory activities (2). The resident FGFR2 kinase in bladder (42), prostate (38, 39), and salivary tumor epithelial cells (34) promotes homeostasis and suppresses the tumor phenotype. This is in contrast to the ectopic FGFR1 that appears in malignant tumors and promotes proliferation and tumorigenesis (1, 2, 5, 38). The resident FGFR2 and ectopic FGFR1 differently phosphorylate FRS2 and activation of the MAP kinase pathway in prostate epithelial cells (2, 30). Currently, we do not know whether the FGFR2 also mediates release of Cks1 from FRS2 or whether activation of the MAP kinase pathway affects release of Cks1 from tyrosine-phosphorylated FRS2 or vice versa in prostate epithelial cells.

In summary, here we report that the release of Cks1 from tyrosine-phosphorylated FRS2 possibly mediates mitogenic signals of the FGFR1 kinase. Determination of whether the four FGFR kinases differ in releasing Cks1 from FRS2 by phosphorylation and whether the release is regulated by other signaling pathways will shed light on understanding the mechanism underlying the FGF signaling pathway.

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