Fibroblast Growth Factor Receptor-1-mediated Endothelial Cell Proliferation Is Dependent on the Src Homology (SH) 2/SH3 Domain-containing Adaptor Protein Crk

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Helena Larsson, Peter Klint, Eva Landgren‡, and Lena Claesson-Weltsh‡

From the Department of Medical Biochemistry and Microbiology, Uppsala University Biomedical Center, Box 575, S-751 23 Uppsala, Sweden

Stimulation of fibroblast growth factor receptor-1 (FGFR-1) expressed on endothelial cells leads to cellular migration and proliferation. We have examined the role of the Src homology (SH) 2/SH3 domain-containing adaptor protein Crk in these processes. Transient tyrosine phosphorylation of Crk in fibroblast growth factor-2-stimulated endothelial cells was dependent on the juxtamembrane tyrosine residue 463 in FGFR-1, and a Crk SH2 domain precipitated FGFR-1 via phosphorylated Tyr-463, indicating direct complex formation between Crk and FGFR-1. Furthermore, Crk SH2 and SH3 domains formed ligand-independent complexes with Shc, C3G, and the Crk-associated substrate (Cas). Tyrosine phosphorylation of C3G and Cas increased as a consequence of growth factor treatment. We examined the role of Crk in FGFR-1-mediated cellular responses by use of cells expressing chimeric platelet-derived growth factor receptor-α/FGFR-1 (αr/FR) wild type and mutant Y463F receptors. The kinase activity of αr/FR Y463F was intact, but both Crk and the adaptor FRS-2 were no longer tyrosine-phosphorylated in the mutant cells. Both wild type and mutant receptor cells migrated efficiently, whereas cells expressing the mutant αr/FR Y463F failed to proliferate and Erk2 and Jun kinase activities were suppressed in these cells. In wild type αr/FR cells transiently expressing an SH2 domain mutant of Crk, Erk and Jun kinase activities as well as DNA synthesis were attenuated. Our data indicate that Crk participates in signaling complexes downstream of FGFR-1, which propagate mitogenic signals.
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**EXPERIMENTAL PROCEDURES**

**FGFR cDNA Constructions**—cDNAs for FGFR-1 (25), PDGFR-α (26), and PDGFR-β (27) were subcloned into the pAlter vector™ (Promega Corp.), and site-directed mutagenesis was performed using the Altered Sites in vitro mutagenesis system (Promega Corp.). A schematic outline of the different receptor constructs used in this study is shown in Fig. 1. The chimeric receptor PDGFR-α/α-FGFR-1 (denoted α/RF wt) was constructed by cleaving the FGFR-1 and PDGFR-α cDNAs with Hin III and Sal I followed by ligation of the fragment corresponding to the extracellular part of PDGFR-α to that corresponding to the intracellular part of FGFR-1 (17). Using the mutagenesis system described, point mutations that changed Tyr-766 or Tyr-463 to phenylalanine residues, or created stop codon including cleavage site for PDGFR-β, were introduced into the respective cDNA using the Altered Sites in vitro mutagenesis system (Promega Corp.). All mutations and constructs were confirmed by nucleotide sequencing. PDGFR-α-FGFR-1 was then engineered to insert domain in FGFR-1 were replaced with the corresponding parts of PDGFR-β, and PDGFR-β cDNAs with HindIII and NruI followed by ligation of the fragment corresponding to the juxtamembrane domain of PDGFR-β into the position of the FGFR-1 endogenous HindIII-NruI fragment. The wild type and the mutated cDNAs were inserted into the eukaryotic expression vector pcDNA I/neo (Invitrogen). All mutations were confirmed by nucleotide sequencing.

**Cell Culture and Transfection**—Primary bovine adrenal cortex capillary endothelial (BCE) cells, kindly provided by Dr. Judah Folkman, Children’s Hospital, Harvard Medical School, Boston, MA, were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% newborn calf serum and 3 ng/ml FGF-2 in 37 °C, 10% CO2. The porcine aortic endothelial (PAE) cell lines were cultured in Ham’s F-12 medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum. The characteristics of PAE cells expressing PDGFRα-PDGFR-β (α/RF wt), PDGFRα-PDGFR-β Y766F (α/RF Y766F), PDGFRα-PDGFR-β Y463F (α/RF Y463F), and FR-1/PR/βKi have been published previously (17, 28). All cell lines used expressed similar levels of receptor proteins and bound the appropriate growth factor with affinities similar to those for wild type receptors. FGF-2 was purchased from Farmitalia Carlo Erba (Milano, Italy), and PDGF-BB was from Peprotech Inc.

**Antisera**—Polyclonal antibodies against Crk II, C3G, and SH-PTP2 were purchased from Santa Cruz Biotechnology, Inc. A mouse monoclonal antibody specific for phosphotyrosine (4G10) was from Upstate Biotechnologies, and an antibody against She was purchased from Transduction Laboratories. Anti-HA antibody was purchased from Roche Diagnostics, Uppsala, Sweden. The rabbit antiserum against phospholipase Cy and the rabbit antiserum against Erk-2 were kind gifts from Dr. Lars Rönnstrand, Ludwig Institute for Cancer Research, Uppsala, Sweden. The rabbit antiserum against FGFR-1 has been described before (28), and a rabbit antiserum specifically reacting with FRS-2 was raised against a peptide corresponding to the C-terminal part of FRS-2.

**Transient Transfection**—PAE cells expressing the wild type chimeric receptor α/RF were cultured in Ham’s F-12 medium supplemented with 10% FCS to 30% confluence. Transfections were done by using SuperFect (Qiagen). For Erk 2 kinase assay, the cells were cultured in T-25 flasks and transfected with 2 μg each of cDNAs encoding HA-Erk and wild type Crk or the Crk SH2 domain mutant in the pCGGS vector. For Jun kinase assay, the cells were cultured in T-25 flasks and transfected with 2 μg each of cDNAs encoding HA-Jun kinase in the pSRα vector and wild type Crk or the Crk SH2 domain mutant. For analysis of labeling index, cells were seeded out on glass placed in 60-mm dishes and transfected with wild type Crk or Crk SH2 domain mutant cDNA using the amount of cDNA needed to get the same amount of Crk expressed in all cells. In all experiments, transfection with only the vector was used as a control. The original Crk II cDNA was kindly provided by Dr. Michiyuki Matsuda (Department of Pathology, National Institute of Infectious Diseases, Tokyo, Japan), the Crk cDNA expressing Crk II SH2 domain mutant was a kind gift from Dr. Kristiina Vuori (Burnham Institute, La Jolla, CA), HA-Erk 2 was from Dr. Ivan Djikic (Ludwig Institute for Cancer Research, Uppsala, Sweden), and HA-Jun kinase was provided by Dr. Par Gerwins (Department of Medical Biochemistry and Microbiology, Uppsala University, Uppsala, Sweden).

**Immunoprecipitation and Immunoblotting**—Cells in 75-cm² flasks were serum starved over night in Ham’s F-12 supplemented with 1% FCS, followed by stimulation with PDGF-BB (100 ng ml⁻¹) or FGF-2 (100 ng ml⁻¹) for 7 min or for different time periods, as indicated, at 37 °C. The monolayers were rinsed with ice-cold phosphate-buffered saline (PBS) containing 100 μM Na2VO4 and lysed for 10 min on ice in 1 ml of Nonidet P-40 lysis buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 300 μM Na2VO4, 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol (DTT)). Lysates were clarified at 10,000 × g for 15 min at 4 °C, and the supernatants were incubated with antibody for 1 h at 4 °C, followed by 1 h of final incubation for 45 min with immobilized protein A (ImmunoSorb; EC Diagnostics, Uppsala, Sweden). The precipitates were washed three times in Nonidet P-40 lysis buffer and twice in PBS containing 100 μM Na2VO4. Sample buffer (0.2 mM Tris-HCl, pH 8.5, 0.5 mM sucrose, 5 mM EDTA, 4% sodium dodecyl sulfate, 0.01% bromphenol blue, and 2% β-mercaptoethanol) was added, and the samples were boiled for 4 min.
at 95 °C before SDS-polyacrylamide gel electrophoresis in 10% gels. For immunoblotting, proteins were electrophoretically transferred onto nitrocellulose membranes (Hybond-C extra, Amersham Pharmacia BioTech). The membranes were blocked in 0.2% Tween 20 in PBS containing 5% bovine serum albumin (BSA). Primary antibody was diluted in PBS. The membranes were incubated with PBS and immunoreactive proteins were visualized by a chemiluminescence detection system based on a protocol described earlier (29). For reprobing the filters, they were stripped in 62.5 mM Tris-HCl, pH 6.7, 2% SDS, and 100 mM β-mercaptoethanol at 50 °C for 30 min. In Vitro Association of GST Fusion Proteins —The SH2 domain of Grb2 was expressed as a part of a GST fusion protein (a kind gift from Dr. J Schlessinger, New York University Medical Center, New York, NY), and used as described earlier (14). The SH2 domain of Crk II and the SH2-SH3 domains of CrkII were also expressed as GST fusion proteins and were kindly provided by Dr. A. Sorokin (Dept. of Medicine and Cardiovascular Research Center, Medical College of Wisconsin, Milwaukee, WI). For association experiments, transfected PAE cells were cultured in 75-cm² flasks and serum starved over night in Ham’s F-12 supplemented with 1% FCS, followed by treatment or not with 0.25% BSA and serum-free medium containing 0.2% FCS was added, and the incubation continued for an additional 24 h. After careful washing in PBS, immunoreactive proteins were visualized by a chemiluminescence detection system based on a protocol described earlier (29).

Peptide Synthesis —The following synthetic peptide, phosphorylated at its tyrosine residue (indicated as pY), was used in this study: pY463, GVSEpYELPEDPRWELPR-COOH. The corresponding nonphosphorylated peptide was dissolved in 50 mM Hepes, pH 7.2, and mixed with 1 ml 1:1 PBS and incubation continued for an additional 24 h. The medium was changed again at day 2 and day 4 (starvation medium), and at the same time PDGF-BB or FGF-2 at different concentrations (0, 1, 10, 20, and 100 ng/ml) were added. As a control, cells were cultured in Ham’s F-12 medium supplemented with 10% FCS. Cell numbers were scored after 5 days. All experiments were performed in triplicate for every concentration of PDGF-BB, and at least two independent cell clones for the chimeric wild type and the mutated (Y463F) receptors were analyzed.

Erk-2 Kinase Assay —After treatment of cells with 100 ng ml⁻¹ PDGF-BB for 7 min at 37 °C, cells were rinsed once with ice-cold PBS containing 100 μM Na₃VO₄ and lysed in lysis buffer (20 mM Hepes, pH 8.0, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1 mM NaF, 100 μM Na₃VO₄, 20 μM leupeptin, 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride, 20 mM Na₃PO₄, 50 mM NaF, 100 μM Na₃VO₄, and 1 mM DTT). Clarified supernatants were incubated with Erk-2 antisera, raised against a C-terminal MAP 2 kinase peptide (EETARFQPQGRYS), end-over-end for 1.5 h at 4 °C. Immobilized protein A (Immunosorb) was added, and the samples were mixed at 4 °C for 30 min. The immune complexes were washed three times in lysis buffer and twice in kinase buffer (20 mM Hepes, pH 8.0, 20 mM MgCl₂, 2 mM MnCl₂, 1 mM DTT) and then incubated for 15 min at 30 °C in 40 μl of kinase buffer containing 10 μg of myelin basic protein (MBP, Sigma) and 5 μCi of [γ⁻³²P]ATP (Amersham Pharmacia Biotech). The kinase reaction was terminated by addition of 40 μl of sample buffer and boiling for 4 min. Samples were run on SDS-PAGE in a 15% SDS-polyacrylamide gel. After fixation in methanol/acetic acid, the gel was dried and analyzed by autoradiography.

Erk 2 kinase activity, in transiently transfected cells expressing HA-Erk 2, wild type Crk, or Crk SH2 domain mutant, was also measured by immunoprecipitation and immunoblotting as described above. Erk 2 was immunoprecipitated by using HA antibodies, and immunoblotting was performed using phosphospecific MAPK antibody (New England Biolabs, Inc.).

JNK Assay —A solid phase assay was used, where c-Jun-(1–79) was expressed as a part of a GST fusion protein and coupled to glutathione-Sepharose 4B. The experiment was performed as described by Gerwins et al. (32). Briefly, chimeric PAE cells were stimulated with 100 ng ml⁻¹ of PDGF-BB or left untreated for 37 °C or left untreated for 4 °C at 37 °C, and then lysed in ice-cold Nonidet P-40 lysis buffer. Clarified lysates were incubated with immobilized GST-c-Jun-(1–79)-Sepharose 4B end-over-end 1 h at 4 °C. The samples were washed twice in Nonidet P-40 lysis buffer and once in kinase buffer (20 mM Hepes, pH 7.5, 0.05% Triton X-100, 2 mM MnCl₂, 10 mM MgCl₂, and 1 mM DTT). The beads were resuspended in kinase buffer supplemented with 10 μCi of [γ⁻³²P]ATP (Amersham Pharmacia Biotech) and used as a sample. The beads were incubated at 30 °C for 20 min and then incubated for 10 min at 37 °C in 40 μl of kinase buffer containing 10 μg of myelin basic protein (MBP, Sigma) and 5 μCi of [γ⁻³²P]ATP (Amersham Pharmacia Biotech). The kinase reaction was terminated by addition of 40 μl of sample buffer and boiling for 4 min. Samples were run on SDS-PAGE in a 15% SDS-polyacrylamide gel. After fixation in methanol/acetic acid, the gel was dried and analyzed by autoradiography.

Jun kinase activity, in transiently transfected cells expressing HA-Jun kinase, wild type Crk, or Crk SH2 domain mutant, was measured by immunoprecipitation and a kinase reaction as above. Jun kinase was immunoprecipitated by using HA antibodies, and GST-Jun-(1–79) was added as a substrate in the kinase reaction.

Labeling Index —PAE cells expressing wild type αR/FR were cultured on coverslips and transiently transfected with wild type Crk and Crk SH2 domain mutant, as described above. The cells were then starved overnight in Ham’s F-12 supplemented with 0.25% BSA and labeled with 1 μCi/ml [³H]thymidine for the last 2 h during culture. The cells were washed, fixed in paraformaldehyde, and covered with autoradiography emulsion (Eastman Kodak Corp.). After 1 week of exposure, the film was developed and unlabeled cells were stained with Mayer’s hematoxylin (Histolab Products AB, Gothenburg, Sweden). The cells were counted, and the results show percentage of labeled nuclei for three different experiments.

RESULTS

Tyrosine-463 in the Juxtamembrane Region of FGFR-1 Is Involved in Mitogenic Signaling —Chimeric PDGFR-α/FGFR-1 (αR/FR) wild type and mutant proteins were ectopically expressed in PAB cells. The cells were stained with antibody against endogenous FGF receptors after different culture times. The PAB cells express low levels of endogenous FGF receptors, but lack expression of PDGF receptors (28, 33). We have previously shown that PAB cells expressing the αR/FR wild type protein migrate and proliferate efficiently in response to PDGF-BB (17). Using this model, we addressed the role of the FGFR-1
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The abilities of PAE cells expressing the wild type aR/FR and cells expressing the mutant aR/FR Y463F to migrate in a mini-Boyden chamber were examined. Cells were seeded on one side of a collagen-coated 8-mm-thick nitrocellulose filter, and the growth factor was suspended in serum-free medium on the other side of the filter. The number of cells that migrated to the other side of the filter during a 6-h incubation was measured. Fig. 3 shows that cells expressing the aR/FR wild type and Y463F mutant migrated with similar efficiencies in this assay, allowing the conclusion that phosphorylation at Tyr-463 is not required for FGFR-1-mediated migration. This is in agreement with our previous data showing that FGFR-1-mediated migration is dependent on a 15-amino acid residue stretch in the C-terminal tail of the receptor (17).

Tyr(P)-463 Is Required for Tyrosine Phosphorylation of FRS-2 and Crk—The adaptor protein FRS-2 has been reported to interact with the FGFR-1 juxtamembrane domain in a phosphotyrosine-independent manner, via a phosphotyrosine binding-like domain in FRS-2. We tested whether the loss of proliferative capacity of PAE cells expressing the aR/FR Y463F mutant to PDGF-BB could be due to decreased FRS-2 tyrosine phosphorylation, and thereby reduced Grb2 binding. Fig. 4A shows that the extent of FRS-2 tyrosine phosphorylation indeed was reduced in cells expressing aR/FR Y463F. Fig. 4B shows that this was not due to a general impairment of FGFR-1 kinase function since PDGF-BB-induced kinase activity of wild type aR/FR and mutant aR/FR Y463F were similar; furthermore, tyrosine phosphorylation of PLCγ, which is known to bind to Tyr(P)-766 in the FGFR-1 C-terminal tail, was induced to the same extent by activation of wild type and mutant chimeric aR/FR (data not shown). In addition, the migration capacity of the Y463F mutant was intact (Fig. 3).

Fig. 4C shows that immunoprecipitation of FRS-2 did not allow detection of co-precipitation of FGFR-1, which is in agreement with previous reports (14). In contrast, immunoprecipitation with Crk II antisera brought down a tyrosine-phosphorylated 150-kDa component after FGF-2 stimulation, which is likely to represent FGFR-1. FGF-induced co-precipitation of FRS-2 in the Crk immunoprecipitate could not be detected (data not shown). The sensitivity of the available FGFR-1 antisera did not allow confirmation that the 150-kDa Crk-associated molecule corresponds to FGFR-1.

The sequence surrounding Tyr-463 (Y-E-L-P) conforms with the reported sequence for binding of the Crk SH2 domain (Y(P)-D-H-P). We used Affi-Gel-immobilized unphosphorylated and phosphorylated Tyr-463-containing synthetic peptides, which were incubated with PAE cell lysates to test whether Crk could bind to this region of FGFR-1. To test for specificity, free unphosphorylated or phosphorylated Tyr-463 peptides were analyzed. The number of wild type PAE cells expressing intact FGFR-1 also increased dose-dependently from 100% (control, serum-starved cells) to 250% for cells treated with 20 or 100 ng/ml PDGF-BB or FGF-2 (0, 1, 10, 20, and 100 ng/ml). The number of cells was counted at day 5 using a Coulter counter. All experiments were performed in triplicate for every concentration of PDGF-BB or FGF-2, and at least two independent cell clones were analyzed. Fig. 3 shows that cells expressing the mutant aR/FR Y463F do not proliferate in response to PDGF-BB. A similar response was seen for PAE cells expressing the mutant aR/FR Y766F). The results show mean ± standard error of the mean (S.E.) of three different experiments.

![Diagram](image.png)

**Fig. 2.** Cells expressing aR/FR Y463F do not proliferate in response to PDGF-BB. Equal numbers of untransfected PAE cells (PAE) and PAE cells expressing aR/FR wt (wt:22), aR/FR Y463F (Y463F:3 and Y463F:5), aR/FR Y766F (Y766F), or FGFR-1 were seeded out in 24-well dishes and cultured in starvation medium (0.25% BSA) for 5 days with or without PDGF-BB or FGF-2 (0, 1, 10, 20, and 100 ng ml⁻¹). The number of cells was counted at day 5 using a Coulter counter. All experiments were performed in triplicate for every concentration of PDGF-BB or FGF-2, and at least two independent cell clones were analyzed. The results show mean ± standard error of the mean (S.E.) of three different experiments.
were mixed with the lysates before incubation with the peptide-coupled Affi-Gel matrix. As seen in Fig. 5A, Crk was retained by the phosphorylated Tyr(P)-463 matrix, but not by the unphosphorylated immobilized Tyr-463 peptides. Addition of free Tyr(P)-463 peptide competed out Crk binding, whereas free unphosphorylated Tyr-463 peptide failed to affect binding of Crk to the immobilized phosphorylated Tyr(P)-463 matrix. To ensure that Crk binding was not dependent on an intermediary component, we incubated a Crk SH2 domain fusion protein (see below) with immobilized phosphorylated and unphosphorylated Tyr-463 peptides. The Crk SH2 fusion protein was retained by the phosphorylated peptide only (data not shown).

To show that Crk is tyrosine-phosphorylated as a consequence of activation of FGFR-1 in intact cells, PAE cells expressing FGFR-1 were with treated for 7 min with FGF-2 (Fig. 5B), which induced a 6-fold increase in Crk tyrosine phosphorylation (Fig. 5B, lanes 1 and 2). To confirm the structural requirement for Crk tyrosine phosphorylation, we examined Crk phosphorylation in PAE cells expressing a series of αR/FR mutants (see Fig. 1 for schematic outline of mutants).
Cells were used that expressed FGFR-1 variants, in which the endogenous juxtamembrane domain (FR-1/PR-bJM), or the kinase insert (FR-1/PR-bK) has been replaced with the corresponding domains from the PDGFR-β. As seen in Fig. 5B, cells expressing FR-1/PR-bKi still responded to FGF-2 treatment whereas in cells expressing FR-1/PR-βJM, FGF-2 stimulation failed to induce an increase in Crk phosphorylation. Fig. 5C (upper panel) shows that the kinetics of Crk tyrosine phosphorylation in FGF-2-stimulated PAE cells was very rapid, with a marked increase occurring already after 1 min of stimulation, indicating that Crk phosphorylation was mediated directly by the FGFR-1. After 30 min of stimulation, the level of Crk tyrosine phosphorylation was back to basal. During this time period, the levels of Crk protein remained unchanged (Fig. 5C, lower panel).

To ensure that endogenous FGF receptors expressed in the PAE cells did not interfere in our analyses, we turned to PAE cells expressing the chimeric αR/FR and mutants of this construct. Fig. 5D shows that cells expressing the αR/FR wild type protein mediated FGF-1-dependent Crk tyrosine phosphorylation in response to PDGF-BB stimulation. In PDGF-BB-stimulated cells expressing αR/FR Y463F, no detectable increase in Crk phosphorylation was observed. In contrast, in cells expressing the αR/FR Y766F mutant, which lacks the binding site for PLCγ, PDGF-BB-induced Crk tyrosine phosphorylation was similar to that in cells expressing the wild type protein.

Crk SH2 and SH3 Domains Mediate Formation of Multiprotein Complexes—Crk is known to couple to a wide spectrum of signal transduction cascades (34). To analyze potential Crk interactions in our cell model, GST fusion proteins covering Crk SH2 or Crk SH2-SH3 domains were coupled to glutathione-Sepharose 4B, and incubated with lysates of PDGF-BB-stimulated and unstimulated PAE cells expressing the αR/FR wild type protein. A number of components were retained on the Crk SH2 and SH2-SH3 matrices, as visualized by SDS-PAGE and immunoblotting using phosphotyrosine antibodies (Fig. 6). Immobilized Crk SH2 domain fusion protein bound the 46-kDa isoform of Shc in a ligand-independent manner. The Crk SH2-SH3 domain fusion protein retained the 54-kDa Shc isoform, as well as the nucleotide exchange factor C3G, as confirmed by blotting with specific antibodies (data not shown), both of which were tyrosine-phosphorylated at basal conditions. The Crk-associated substrate (Cas) also bound to the Crk SH2-SH3 domain. Tyrosine phosphorylation of both C3G and Cas were increased after ligand stimulation. In some experiments, the Crk SH2-SH3 domain fusion protein also retained the tyrosine phosphatase SHP-2, which was phosphorylated at increased levels after growth factor treatment. Binding of the nucleotide exchange factor Sos was not detected, in accordance with previous studies where such interactions have been difficult to identify. The adaptor FRS-2 was also not detected in these analyses (data not shown).

Fig. 6C shows that the Crk SH2 domain pulled down a 150-kDa component from PAE cells expressing FGFR-1 or the αR/FR chimeric receptor after appropriate growth factor treatment. This molecule was not precipitated by the Crk SH2 domain fusion protein from cells expressing the αR/FR Y463F cells, indicating that the 150-kDa component indeed corresponds to the receptor. Direct confirmation by immunoprecipitation with FGRF-1 antiserum was not possible using available reagents. GST alone failed to bring down tyrosine-phosphorylated material (Fig. 6C).

Loss of Erk and Jun Kinase Activation in the Y463F Mutant Cells—Previous reports have demonstrated SH3-domain-dependent association between Crk and nucleotide exchange factors such as Sos (coupling to the MAP kinase cascade) and C3G (coupling to the Jun kinase cascade) (35–38), the latter of which was associated with the Crk SH2-SH3 domain fusion protein and tyrosine-phosphorylated in response to activation of the αR/FR in our cell model (see above). We examined the effect of the Y463F mutation in FGFR-1 on activation of the MAPK kinase Erk2 and of the Jun kinase in PAE cells expressing wild type αR/FR or mutant αR/FR Y463F. Erk2 was immunoprecipitated and incubated in the presence of [γ-32P]ATP and MBP. As seen in Fig. 7A, the level of MBP phosphorylation as
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**DISCUSSION**

We show in this paper that Crk is tyrosine-phosphorylated by FGFR-1 and that stable complex formation between the Crk SH2 domain and FGFR-1 is dependent on phosphorylated Tyr-463 in the receptor juxtamembrane domain. Thus far, FGFR-1 has been shown to associate in stable complexes only with PLCγ, whereas other signal transduction molecules such as Src, Shb, and FRS-2 are tyrosine-phosphorylated without stable complex formation with the activated dimerized receptors (39).

We used PAE cells expressing chimeric PDGFR-α/FGFR-1 wild type and mutant proteins for these studies. PAE cells have been demonstrated to faithfully reproduce signal transduction events identified in primary cells (40). In order to avoid interference of endogenous FGF receptors in these cells, we have employed chimeric receptors and we show in this paper (Figs. 2 and 3), and have shown previously that signals for migration and proliferation are efficiently transduced via the chimeric receptor (17). In contrast, the mutant αR/FR Y463F fails to mediate proliferative signals. The amino acid sequence surrounding Tyr-463 (Y-E-L-P) agrees well with the one assigned by Songyang et al. (41) as the preferred binding-motif for the Crk SH2 domain. Similar motifs have been implicated in binding of other SH2 domain-containing signal transduction pro-
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We have previously reported that the FGFR-1 Y463F mutant expressed in L6 myoblasts mediates intact activation of Raf (14), and Mohammadi et al. (13) have shown that an FGFR-1 mutant lacking four tyrosine phosphorylation sites, including Tyr-463, mediates increased incorporation of [3H]thymidine similar to the wild type FGFR-1. However, PAE cells expressing the αR/FR Y463F mutant receptor failed to proliferate in response to growth factor. By employing chimeric receptors, we have ensured that exogenous or endogenous FGF-2 sources do not affect the outcome of the experiment. Most cells in tissue culture express FGF receptors and produce FGF. Furthermore, transient overexpression of wild type Crk, as well as the Crk SH2 domain mutant to similar protein levels (data not shown), obliterated growth factor-induced DNA synthesis in the αR/FR PAE cells (Fig. 9). The effect of the Crk SH2 domain mutant is likely to depend on saturation of downstream signal transduction components, thereby inhibiting endogenous normal wild type Crk function. The effect of overexpression of wild type Crk could in part be due to displacement of FRs-2, or to abortion of downstream signaling by saturating binding to signal transduction proteins, such as Sos and C3G. Dependent on the relative expression levels of receptors and signal transduction molecules, overexpression of wild type versions of signal transduction molecules may suppress downstream signal transduction as described previously (47).

The nucleotide exchange factor C3G is structurally related to Sos within the catalytic domain and Sos as well as C3G contains multiple proline-rich domains which interact with the Crk SH3 domain (36). Whereas Sos regulates Ras activity, C3G has been reported to activate Rap1/smegg21/Krev-1 (48), a Ras-related GTPase, which counteracts the effects of Ras in transformation (49). Rap1 appears to transduce signals that regulate the kinetics of Erk 1/2 activation, possibly in a cell type- and stimulus-dependent manner (50, 51). The Crk/C3G complex has also been shown to activate Jun kinase (37), by a Ras-independent mechanism (38). The Jun kinase is classically activated by stress stimulation, such as UV irradiation, hyperosmolarity and inflammatory cytokines (52) via a pathway involving the recently identified MEKK1–4 (32, 53) and the downstream MKK4 and MKK7 (54, 55). This pathway has been shown to transduce signals for apoptosis (52) although Jun kinase appears to function also in other cellular responses and may also protect against apoptosis. Crk has been shown to promote apoptosis in Xenopus eggs, and immunodepletion of Crk inhibited apoptosis (56). We show that obstruction of Crk signal transduction downstream of the FGFR-1 attenuated activation of Jun kinase, in agreement with the report by Tanaka et al. (37). Thus, it is possible that FGFR-1 transduces both positive and negative signals and that the final read-out is dependent on the balance between these signals. The contribution of Jun kinase to FGFR-1-dependent cellular responses remain to be identified.

Crk has recently been shown to be a substrate for the PDGF α- and β-receptors (23), although without apparent consequence for PDGF-induced biological responses. Tanaka and co-workers used the pheochromocytoma cell line PC12 to analyze the role of Crk in neuronal differentiation. Microinjection of Crk induced neurite formation, which was blocked by point mutation in either of the Crk SH2 or SH3 domains (57). Moreover, data recently reported by York et al. (24) indicated Rap1 in neuronal cell differentiation. One may infer from these studies that Crk is involved in a multitude of cellular responses, probably by virtue of its participation in signal transduction pathways gated via Ras and Ras-related proteins. We will focus our further studies on the role of signal transduction via Ras proteins, such as Nck (Y-D-E-P), Abl (Y-E-N-P) and SHP-2 (Y-I-V-X-P) (41). We were interested in the possibility that the adaptor molecule Nck is a substrate for FGFR-1; if this is the case, Nck may compete with Crk for interaction with the same site on FGFR-1. Nck is a widely expressed adaptor molecule, containing one SH2 and three SH3 domains (42), which causes trans-formation of fibroblasts and tumor formation in nude mice (43, 44). Nck has been reported to participate in FGFR-1 signal transduction in mesoderm induction during Xenopus development (45). We failed to detect FGFR-1-dependent tyrosine phosphorylation of Nck in PAE cells (data not shown). Furthermore, overexpression of wild type Nck or an SH2 domain mutant of Nck still allowed increased DNA synthesis in response to growth factor treatment, although basal labeling index was increased (data not shown). Thus, our data do not show a role for Nck in FGFR-1-mediated proliferation in the PAE cells.

Interactions between Crk and two guanine nucleotide exchange factors, C3G and Soc, have been identified (46). It is well established that the Grb2/Sos complex mediates activation of Ras, which couples to a cascade of serine/threonine kinases (Raf, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase, and Erk1/Erk2). We show decreased Erk2 activation in cells expressing the Y463F mutant receptor, and in cells overexpressing a Crk SH2 domain mutant. The recently characterized FGFR-1 substrate FRS-2 (18) presents four binding sites for Grb2 and is therefore an important mediator of Ras activation. FRS-2 was not appreciably tyrosine-phosphorylated in cells expressing mutated Y463F receptors, and we infer that the reduction in Erk2 activation and proliferative capacity of the αR/FR Y463F cells is due to both loss of Crk and FRS-2 signal transduction. FRS-2 has been shown to associate with the unstimulated FGFR-1 via its juxtamembrane domain in a phosphotyrosine-independent manner, using the yeast two-hybrid screen. Our data indicate that removal of Tyr-463 in FGFR-1 leads to a loss of FRS-2 adaptor function.

Fig. 9. Overexpression of wild type and mutant Crk interferes with FGF-2-induced DNA synthesis. Labeling index was analyzed in PAE cells expressing αR/FR wt transiently transfected with wild type Crk or Crk SH2 domain mutant. The cells were cultured in Ham's F-12 supplemented with 10% FCS and labeled with 1 μCi/ml [3H]thymidine for the last 2 h during culture. The cells were fixed, washed, and processed for autoradiographic determination of [3H]thymidine incorporation. The results show mean percentage of labeled nuclei of total cells ± standard error of mean (S.E.) for three determinations.
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