The Cytoplasmic Tyrosine Kinase Pyk2 as a Novel Effector of Fibroblast Growth Factor Receptor 3 Activation*

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Receptor tyrosine kinases comprise nearly two dozen different families of homologous proteins in humans, with at least 40 distinct members, and are responsible for the integration of many different signals that affect development, proliferation, and homeostasis. In the fibroblast growth factor receptor (FGFR) family, four homologous receptors have been identified, designated FGFR1, FGFR2, FGFR3, and FGFR4. The basic structure of FGFRs consists of three extracellular Ig-like domains stabilized by characteristic disulfide bonds, a single membrane-spanning segment, and an intracellular split tyrosine kinase domain (1, 2). Fibroblast growth factors, a large family of at least 20 growth factors, act in concert with heparan sulfate proteoglycans as high affinity agonists that induce FGFR dimerization and transphosphorylation (3–6). Activation of FGFRs controls an array of biological processes, including cell growth, differentiation, migration, wound healing, and angiogenesis. FGFRs and their ligands play a major role in autocrine and paracrine signaling loops that have been implicated in a number of malignancies, including cancers of the stomach, breast, thyroid, prostate, and pancreas and also some leukemias (7, 8).

A frequent translocation observed in multiple myeloma, t(4;14)(p16.3;q32.3), involves the FGFR3 gene and results in increased expression of FGFR3 alleles that contain activating mutations (9–11). This translocation occurs with an incidence of about 25% in multiple myeloma and places the FGFR3 gene located at 4p16.3 in proximity with the 3′ IgH enhancer at 14q32.3 leading to significant FGFR3 overexpression (9). Furthermore, mutations in FGFR3 were identified in the overexpressed, translocated alleles of several tumors and cell lines. The identified mutations, Y373C and K650E, correspond to germline FGFR3 mutations that cause the lethal skeletal syndromes TDI and TDII, respectively (12, 13). Expression of FGFR3 has also been reported in human bladder and cervical carcinomas (14), including activated mutants corresponding to the mutations R248C, S249C, G370C, and K650E, previously identified as causing TDI or TDII (12, 13). Thus, expression of a constitutively activated FGFR3 may be an important event in aberrant signal transduction in a variety of human neoplasias.

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In this study we demonstrate a novel interaction between FGFR3 and Pyk2 in human 293T cells and also in the multiple myeloma cell line U266.2. In these experiments, hyperactivation of FGFR3 led to increased tyrosine phosphorylation of Pyk2. Previous work from our lab has demonstrated the activation of Stat1, Stat3, and Stat5 in response to FGFR3 activation (27–29). Given the importance of Stat activation in a variety of malignancies (30), we surveyed Stat1, Stat3, and Stat5 for altered activation in response to the coexpression of activated FGFR3 with Pyk2. These experiments demonstrated a significant interaction between FGFR3 and Pyk2 signaling pathways in regulating the activation of Stat5.

EXPERIMENTAL PROCEDURES

**FGFR Constructs**—The full-length FGFR3 wild type, kinase-active (TD/K560E), and myristoylated forms of FGFR3 have been previously described (31, 32). The FGFR3–C-term was constructed by inserting a linker from the XhoI site in FGFR3 (Leu457-Glu458) to the downstream XbaI site in the pcDNA3 vector of full-length FGFR3. The linker contained a stop codon leading to the truncation of the protein at residue 459. The FGFR3–JM clone was constructed from a FGFR3–C-term derivative. First the plasmid was digested with XhoI and the ends were filled in with Klenow polymerase fragment to create blunt ends. It was then ligated with HindIII and ligated to a HindIII-Stul fragment containing the N terminus and transmembrane domain of FGFR3. The protein is truncated at residue 410. The full-length FGFR2, FGFR1, and FGFR4 constructs have been described previously (29, 33).

Pyk2, Shp2, and GFP-Stat5B Constructs—Myc-Pyk2 wild type, Y402F, and K457A have been described previously (34). To construct the PRNK clone, the C terminus of Pyk2 was amplified after the kinase domain at the first Met residue, Met365. The PCR product was digested at the 5′ end with BamHI and at the 3′ end with XbaI and cloned into the BglII and XbaI sites on the pCS-Myc tag vector. It was subcloned by Dr. Gen-Sheng Feng of the Burnham Institute (La Jolla, CA). The PGK-Stat5B clone was generously provided by Dr. Christina Carter-Su (35).

Antibodies—Antibodies were obtained from the following sources: Myc(E9E10), FGFR3 (C-15), FGFR3 (H-109), Fig (C-15, FGFR1), Bek (C-17; FGFR2), FGFR4 (C-16), SH-PTP2 (C-18; Shp2), Pyk2 (C-19), GFP(FPL), Stat5 (C-17), horseradish peroxidase donkey anti-goat (Santa Cruz Biotechnology), 4G10 (anti-phosphotyrosine; Upstate Biotechnology), G4D (anti-phospho-tyrosine; Upstate Biotechnology, Inc.), Pyk2 (5906; Ref. 34); and horseradish peroxidase anti-mouse and horseradish peroxidase anti-rabbit (Amersham Biosciences).

**Immunoprecipitation and Immunoblot**—293T cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and incubated at 37°C in 10% CO2. Subconfluent cells were transfected with 0.5–10 μg of DNA by calcium phosphate precipitation (36). Two days after transfection, the cells were harvested and lysed in 1% Nonidet P-40 lysis buffer (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin) in the presence of phosphatase inhibitors. The lysates were immunoprecipitated with antibodies for 2 h at 4°C. Protein A-Sepharose beads were added and incubated for 2 h at 4°C. The immunoprecipitated samples were washed three times with lysis buffer, boiled for 5 min in sample buffer, and separated by 12.5% SDS-PAGE. The proteins were transferred to Immobilon-P membranes (Millipore) and blocked in 5% milk, Tris-buffered saline, 0.05% Tween 20 or in 3% bovine serum albumin, Tris-buffered saline, 0.05% Tween 20 (for anti-phosphotyrosine blots). The membranes were immunoblotted with antibodies at room temperature for 2–3 h or overnight at 4°C. After primary incubation the membranes were washed with Tris-buffered saline, 0.05% Tween 20 and incubated with horseradish peroxidase-conjugated secondary antibodies. The proteins were detected by ECL (Amersham Biosciences). To reprobe with other antibodies, the membranes were stripped of bound antibodies in stripping buffer (90 mM 2-mercaptoethanol, 2% SDS, 50 mM Tris-HCl, pH 6.8) and incubated for 1 h at 80°C.

**Endogenous Association**—For endogenous Pyk2 association with FGFR3, the human multiple myeloma cell line U266.2 (9, 37, 38) was used (gift from P. Leif Bergsagel). The cells were maintained in RPMI 1640 with 5-glutamine medium (Cellgro) supplemented with 10% fetal bovine serum (heat-inactivated) and penicillin/streptomycin. Lysate was prepared in 1% Nonidet P-40 lysis buffer. 2.5 mg of total protein was immunoprecipitated with anti-FGFR3/C-15 as described above. Endogenous Pyk2 was detected with anti-Pyk2 (5906) on the immunoblot.

**RESULTS**

Interaction of FGFR3 and Pyk2—Fig. 1A presents schematically the essential features of the FGFR3 derivatives (Fig. 1A) and Pyk2 derivatives (Fig. 1B) utilized in this study. Following transfection into 293T cells, FGFR3 was readily detected by immunoblotting of Pyk2 immunoprecipitates collected using the monoclonal antibody 9E10 against the Myc tag (Fig. 2A, top panel). Reciprocally, Pyk2 was readily detected by immunoblotting of FGFR3 immunoprecipitates (Fig. 2A, bottom panel). Pyk2 associates equally well with either WT or constitutively activated FGFR3 with the K650E mutation (TD) responsible for the neonatal lethal developmental syndrome thalassemic dysplasia type II (13) (Fig. 2A, lanes 5 and 6). Similarly, this association is not dependent on the kinase activity of FGFR3, as shown by coimmunoprecipitation of kinase-dead (KD) FGFR3 with Pyk2 (Fig. 2A, lane 3).

To examine further whether tyrosine phosphorylation of FGFR3 is required for interaction with Pyk2, FGFR3 phosphorylation site add-back mutants (28) retaining only the single sites of tyrosine phosphorylation Tyr770, Tyr785, Tyr856, or Tyr870 were also found to interact with Pyk2 in coimmunoprecipitation experiments, as did the quadruple mutant myr-FGFR3[4F] with all four sites mutated simultaneously to Phe (data not shown). In fact, the entire kinase domain of FGFR3 was difficult to immunoprecipitate experiments, as did the quadruple mutant myr-FGFR3[4F] with all four sites mutated simultaneously to Phe (data not shown). In fact, the entire kinase domain of FGFR3. As shown in Fig. 2B, lane 3). Deletion of the extracellular and transmembrane domains and attachment of an N-terminal myristoylation signal to create a biologically active myr-FGFR3 derivative (32) also had no effect on the association of Pyk2 with FGFR3 (Fig. 2A, lanes 2–4). Together, these observations lead us to conclude that Pyk2 is binding within the intracellular juxtamembrane domain of FGFR3. To confirm this, most of the juxtamembrane domain was deleted in the FGFR3–C-term clone, thereby creating FGFR3–JM, which was cotransfected with Pyk2. The interaction between Pyk2 and FGFR3 was disrupted with this clone (Fig. 2B, lane 4). This suggests that the major site of Pyk2 association is within the juxtamembrane region of FGFR3.

To examine the region of Pyk2 required for the interaction with FGFR3, three different Pyk2 deletion mutants were utilized. The ΔFERM construct has residues 39–364 deleted to remove the FERM domain. The ΔKIN construct has a C-terminal truncation after residue 413 that removes the kinase domain as well as the Pro-rich and paxillin-binding domains. The PRNK clone is a naturally occurring splice variant of Pyk2 in which the FGFR3 and kinase domains are absent, although the Pro-rich and Pax domains are retained (Fig. 1B) (39). These Pyk2 derivatives were transfected into 293T cells with or without full-length FGFR3–WT. Cell lysates were then used to prepare Pyk2 immunoprecipitates using α-Myc(9E10) monoclonal antibody and subsequently immunoblotted for the presence of FGFR3. As shown in Fig. 2C (top panel), only Pyk2-WT and the ΔFERM construct were able to coimmunoprecipitate
FGFR3 constructs. A schematic of the receptor tyrosine kinase FGFR3 depicts: the extracellular domain (ECD), transmembrane domain (TM), intracellular juxtamembrane domain (JM), and kinase domain (KIN). The activating K650E mutation (TD) and the kinase-dead K508R mutation (KD) are also indicated. The myr-FGFR3 derivatives lack the extracellular domain and transmembrane domain and are targeted to the plasma membrane by an N-terminal myristoylation signal (myr). FGFR3–ΔC-term has a stop codon introduced leading to truncation at residue 459, and deletion of the kinase domain. FGFR3–ΔJM is truncated after residue 459. B, Pyk2 constructs. A schematic of the cytoplasmic tyrosine kinase Pyk2 depicts: the FERM domain, the kinase domain (KIN), proline-rich regions (PR), and paxillin-binding domain (PAX). Also indicated are Tyr662, important for c-Src binding (19); Tyr402, important for c-Grb2 binding (15, 22–24); and the kinase-inactive K457A mutation (KD) (15). The PRNK derivative is a splice variant of Pyk2 that leads to initiation of the FERM domain, the kinase domain, the FERM, the FERM and PRNK, and paxillin-binding domain, transmembrane domain, kinase-dead K508R, and kinase-dead K508E mutation (KD) are also indicated. The myr-Pyk2 derivatives lack the extracellular domain and transmembrane domain and are targeted to the plasma membrane by an N-terminal myristoylation signal (myr). Pyk2 at detectable levels (data not shown).

Pyk2 Association with Other FGFR Family Members—In addition to FGFR3, we were interested to determine whether Pyk2 binds to other members of the FGFR family. To this effect, 293T cells were transfected with Myc-tagged Pyk2 and full-length FGFR1, FGFR2, FGFR3, or FGFR4. The cell lysates were then immunoprecipitated with antisera to Myc(9E10) or FGFR3 (C-15) to examine FGFR3 expression levels. FGFR3 expression was also examined in α-FGFR3 (H-100) immunoprecipitates, which were then immunoblotted with α-FGFR3 (H-100). FGFR3 and may play a role in signaling pathways specific to these two members of the FGFR family.

Association of Endogenous FGFR3 and Pyk2—To examine whether the endogenous proteins FGFR3 and Pyk2 interact, we chose the multiple myeloma cell line UTMC-2, which expresses both proteins. As shown in Fig. 4, we were able to detect the presence of Pyk2 by immunoblotting of α-FGFR3 immunoprecipitates prepared from lysates of UTMC-2 cells (Fig. 4, lane 2). This confirms that the interaction of FGFR3 and Pyk2 can occur between endogenous proteins and is not
membrane was cut and immunoblotted (IB) to examine FGFR expression levels. Pyk2. The membrane was stripped and reprobed with anti-FGFR3.

2.5 mg of total protein was immunoprecipitated (IP) with a-Myc(9E10). The cells were lysed in 1% Nonidet P-40 lysis buffer. Multiple myeloma cell line UTMC-2 (37) was obtained from P. Leif Bergsagel (9, 38). The cells were lysed in 1% Nonidet P-40 lysis buffer.

Because Pyk2 is regulated by tyrosine phosphorylation, FGFR3 Activation Leads to Increased Pyk2 Phosphorylation—Because Pyk2 is regulated by tyrosine phosphorylation, we examined Pyk2 for changes in phosphotyrosine content when coexpressed with FGFR3. 293T cells were transfected with FGFR3 derivatives (WT, TD, or KD) along with Pyk2 derivatives (WT, kinase-dead K457A, or c-Src-binding mutant Y402F), and Pyk2 was examined for phosphotyrosine (Fig. 5). The activated FGFR3-TD mutant, in comparison with FGFR3-WT, resulted in a modest increase in tyrosine phosphorylation of Pyk2-WT (Fig. 5, lanes 4 and 7). However, the activated FGFR3-TD mutant led to a significant increase in the tyrosine phosphorylation of the c-Src-binding mutant Pyk2-Y402F, which exhibited only negligible phosphorylation in response to FGFR3-WT (Fig. 5, lanes 6 and 9). This phosphorylation was dependent upon the presence of a functional Pyk2 kinase domain, as indicated by the Pyk2-K457A mutant (Fig. 5, lanes 5, 8, and 11). These results demonstrate the ability of FGFR3 to increase the tyrosine phosphorylation of Pyk2, even when c-Src recruitment is abrogated.

FIG. 4. Endogenous FGFR3 and Pyk2 association. The human multiple myeloma cell line UTMC-2 (37) was obtained from P. Leif Bergsagel (9, 38). The cells were lysed in 1% Nonidet P-40 lysis buffer. 2.5 mg of total protein was immunoprecipitated (IP) with a-Myc(9E10) and then immunoblotted (IB) with anti-PYK2 (5906) (34) to detect endogenous Pyk2. The membrane was stripped and reprobed with anti-FGFR3.

Because of the reported involvement of Shp2 in antagonizing FGFR3 activation, we next examined the recovery of Shp2 in complexes with Pyk2. Cell lysates expressing Pyk2, FGFR3, and Shp2 were immunoprecipitated with 9E10 to recover Myc-tagged Pyk2, and immunoprecipitated proteins were then immunoblotted to detect Shp2 or FGFR3. The association of Shp2 with Pyk2 was readily detected using Shp2-WT (Fig. 6A, lanes 3–7) but was enhanced when the phosphatase-inactive Shp2 C459S mutant was coexpressed together with FGFR3, either WT or TD (lanes 8 and 9). The presence of FGFR3 complexed with Pyk2 was readily detected in the absence or the presence of coexpressed Shp2-WT (lanes 3–6) but was significantly depressed by coexpression with the phosphatase-inactive Shp2 C459S mutant (lanes 8 and 9). The increase in association of the Shp2(CS) mutant is consistent with the substrate trapping ability of Cys to Ser mutations within the signature motif of protein-tyrosine phosphatases (42–44). These results suggest that the association of Pyk2 with either FGFR3 or Shp2 may be mutually exclusive.

This was further explored by examining Shp2 or FGFR3 immunoprecipitates for the presence of associated Pyk2 by immunoblotting. Pyk2 was present in Shp2 immunoprecipitates, irrespective of the presence of coexpressed FGFR3 (WT or TD) (Fig. 6B, middle panels). FGFR3 was largely undetectable in Shp2 immunoprecipitates under these conditions. Reciprocally, Pyk2 was present in FGFR3 immunoprecipitates, irrespective of the presence of coexpressed Shp2 (Fig. 6B, lower panels). Shp2 was undetectable in FGFR3 immunoprecipitates under these conditions. Although we cannot completely rule out the possibility of the existence of the tertiary complex at levels too low for detection, these results suggest that Pyk2 may form mutually exclusive complexes either with the tyrosine kinase FGFR3 or with the tyrosine phosphatase Shp2.

Shp2 C459S mutant (40) (Fig. 6A, lanes 5–10). The increase in Pyk2 phosphorylation in the presence of FGFR3-TD was greatest when coexpressed with the phosphatase-inactive Shp2 mutant (lane 9) and was least when coexpressed with excess Shp2-WT (lane 6). These results indicate that FGFR3 activation leads to an increase in phosphorylation of Pyk2 and that this increased tyrosine phosphorylation is antagonized by the tyrosine phosphatase Shp2.

Pyk2 Association with FGFR3 and Shp2—Previous work by others has shown the direct association of Shp2 with Pyk2 (26, 41). We next examined the recovery of Shp2 in complexes with Pyk2. Cell lysates expressing Pyk2, FGFR3, and Shp2 were immunoprecipitated with 9E10 to recover Myc-tagged Pyk2, and immunoprecipitated proteins were then immunoblotted to detect Shp2 or FGFR3. The association of Shp2 with Pyk2 was readily detected using Shp2-WT (Fig. 6A, lanes 3–7) but was enhanced when the phosphatase-inactive Shp2 C459S mutant was coexpressed together with FGFR3, either WT or TD (lanes 8 and 9). The presence of FGFR3 complexed with Pyk2 was readily detected in the absence or the presence of coexpressed Shp2-WT (lanes 3–6) but was significantly depressed by coexpression with the phosphatase-inactive Shp2 C459S mutant (lanes 8 and 9). The increase in association of the Shp2(CS) mutant is consistent with the substrate trapping ability of Cys to Ser mutations within the signature motif of protein-tyrosine phosphatases (42–44). These results suggest that the association of Pyk2 with either FGFR3 or Shp2 may be mutually exclusive.

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Stat5 Activation by FGFR3 Is Regulated by Pyk2—Because many studies have demonstrated constitutive activation of
the lysates were immunoprecipitated with Myc(9E10) antibody and immunoblotted with 4G10, Pyk2(C-19), FGFR3(C-15), and Shp2(SH-PTP2 C-18) antisera. The lysates were also examined for Shp2 and FGFR3 expression. B, FGFR3 and Shp2 form separate complexes with Pyk2. 293T cells were transfected with: Myc-tagged Pyk2, myr-FGFR3 derivatives WT or TD, and Shp2-WT or CS mutants. The cell lysates were immunoprecipitated with Pyk2, myr-FGFR3 derivatives WT or TD, and Shp2-WT. Equal amounts of lysates were immunoblotted with 4G10, Pyk2(C-19), FGFR3(C-15), and Shp2(SH-PTP2 C-18) antisera. The lysates were examined for expression levels of Pyk2, Shp2, and FGFR3.

**Fig. 7.** Shp2 and FGFR3-mediated Pyk2 phosphorylation. A, phosphorylation of Pyk2 mediated by FGFR3 and Shp2. 293T cells were transfected with Myc-tagged Pyk2, myr-FGFR3 derivatives WT or TD and Shp2-WT or CS mutant. The cell lysates were immunoprecipitated with Myc(9E10) antibody and immunoblotted with 4G10, Pyk2(C-19), FGFR3(C-15), and Shp2(SH-PTP2 C-18) antisera. The lysates were also examined for Shp2 and FGFR3 expression. B, FGFR3 and Shp2 form separate complexes with Pyk2. 293T cells were transfected with: Myc-tagged Pyk2, myr-FGFR3 derivatives WT or TD, and Shp2-WT. Equal amounts of lysates were immunoprecipitated (IP) with Shp2 or FGFR3 antisera. Immunoblot (IB) analysis was performed using Pyk2 (C-19), SH-PTP2 (C-18), and FGFR3 (C-15) antisera. The lysates were examined for expression levels of Pyk2, Shp2, and FGFR3.

Stats in a large number of tumor cell lines, including blood malignancies and solid tissues (30), we examined Stat family members for activation in response to FGFR3 and Pyk2 expression. Tyrosine phosphorylation on Stats is required for activation and translocation to the nucleus. After examining the phosphotyrosine of endogenous Stat1, Stat3, Stat5, and Stat6, only Stat5 showed a modulation of activation with Pyk2 and FGFR3 coexpression (data not shown). In addition, our lab previously demonstrated a relationship between activated FGFR3 and Stat5 activation (27).

To characterize Stat5 activation more fully, we transfected a GFP-Stat5B expression construct (35). We examined Stat5 for tyrosine phosphorylation in cell lysates transfected with Pyk2 derivatives, FGFR3 derivatives, and GFP-Stat5B. As seen in Fig. 7A (top panel), a dramatic increase in phosphotyrosine is seen with activated FGFR3 expression, in either the presence (lane 5) or the absence (lane 7) of overexpressed Pyk2-WT. Significantly, increased tyrosine phosphorylation of GFP-Stat5B in response to activated FGFR3 was decreased in the presence of the dominant negative Pyk2-K457A mutant (lane 10). Interestingly, Pyk2-WT is able to activated Stat5 alone and is dependent on its kinase activity (lanes 3 and 8).

The importance of c-Src association with Pyk2 in the activation of GFP-Stat5B was examined in Fig. 7B using the Y402F mutant of Pyk2. As seen in lane 4, tyrosine phosphorylation of GFP-Stat5B is dependent on c-Src recruitment. Activated FGFR3 is able to compensate for the Y402F mutant in GFP-Stat5B activation (lane 5). This implies that activated FGFR3 overcomes the normal regulation of Pyk2 by autophosphorylation.

In Fig. 7C, the phosphotyrosine on GFP-Stat5B was quantitated relative to the expression of GFP-Stat5B. The coexpression of FGFR3-TD and Pyk2-WT does not lead to a significant increase in phosphorylation over FGFR3-TD alone (lanes 4 and 6). The kinase-dead Pyk2 is able to partially block the downstream signaling from FGFR3 (compare lanes 4 and 8). Interestingly, the mutation Y402F in Pyk2 leads to an increase of Stat5B activation when coexpressed with FGFR3-TD (lane 10).

Examination of Fig. 7 reveals changes in tyrosine phosphorylation of GFP-Stat5B in response to the expression of Pyk2 or FGFR3 alone, or expressed together, that are suggestive but not definitive concerning an interaction between these proteins. However, the decrease of phosphotyrosine on GFP-Stat5B, when the dominant negative Pyk2-K457A mutant is coexpressed with the activated FGFR3-TD mutant, provides compelling evidence of a significant interaction between FGFR3 and Pyk2 signaling pathways in the activation of Stat5.

**DISCUSSION**

Activation of FGFR3 has been shown previously to result in activation of mitogen-activated protein kinase and phosphatidylinositol 3-kinase signaling, phosphorylation of phospholipase Cγ and Shc, increased Bcl-XL expression, activation of Stat1, Stat3, and Stat5, and recruitment of the adapter protein SH2-B (27, 28, 45–48). However, association with Pyk2 or its relative focal adhesion kinase has not been previously reported. We demonstrate here that Pyk2 and FGFR3 can be recovered in a complex and that this association is mediated by the juxtamembrane domain of FGFR3 and the kinase domain of Pyk2 (Fig. 2). Within the FGFR family, we found that FGFR2 binds Pyk2 at a level comparable with that of FGFR3. However, association of Pyk2 with either FGFR1 or FGFR4 was not observed. At this time we cannot rule out the possibility that FGFR1 and FGFR4 may bind to Pyk2 because of the lower levels of expression in Fig. 3, yet the results presented here indicate that the association with Pyk2 may be specific to FGFR2 and FGFR3. FRS2, FGFR substrate 2, has been implicated in downstream signaling from activated FGFR1 and binds Grb2 and Shp2 substrates (49, 50). The observed association of Pyk2 with the juxtamembrane domain of FGFR3 is similar in some respects to the observation that FRS2 binds to the juxtamembrane domain of FGFR1 (49, 50), providing for recruitment of Grb2-SOS complexes that couple to Ras/mitogen-activated protein kinase proliferative pathways (51–53). Although most of our experiments were carried out under conditions of overexpression for both Pyk2 and FGFR3, using 293T cells, we were also able to demonstrate association of endogenous Pyk2 and FGFR3 in the human multiple myeloma cell line UTERM-2 (Fig. 4).

We observed that expression of activated FGFR3 leads to increased phosphorylation of Pyk2 (Fig. 5). The increased phosphorylation of Pyk2 in the presence of the activated FGFR3-TD mutant (but not FGFR3-WT) was still observed for the mutant
Pyk2-Y402F (Fig. 5), which lacks a major autophosphorylation site that recruits c-Src to activated Pyk2. Thus, we conclude that the FGFR3-stimulated increase in tyrosine phosphorylation of Pyk2 is independent of Pyk2 phosphorylation at Tyr402 and the resulting recruitment of c-Src to these signaling complexes. Of course, the observation of increased Pyk2 phosphorylation in the presence of hyperactivated FGFR3 raises the question whether FGFR3 may directly phosphorylate Pyk2, an issue that we are currently examining.

We also examined the role of Shp2 in regulating tyrosine phosphorylation of Pyk2. We demonstrated that coexpression of a phosphatase-dead Shp2 increases the observed phosphorylation of Pyk2 in response to hyperactivated FGFR3 (Fig. 6A). This indicates that Shp2 activity is important as a negative regulator of Pyk2 tyrosine phosphorylation.

The demonstration that Pyk2 can be recovered in complexes with FGFR3 or in complexes with Shp2 (Fig. 6B) suggests two distinct but equally important functions for FGFR3 in associating with Pyk2. One function would be to increase the extent of Pyk2 phosphorylation, contributing to the activation of downstream signaling pathways. Because Shp2 can reduce phosphorylation of Pyk2, a separate function for FGFR3 may be to sequester Pyk2 from the phosphatase Shp2, leading to prolonged Pyk2 signaling.

To identify a biological consequence of the interaction between FGFR3 and Pyk2, we examined the activation of Stat proteins. Many studies have demonstrated constitutive activation of Stats in a large number of tumor cell lines (30), and our lab has previously demonstrated a relationship between activated FGFR3 and the activation of Stat1, Stat3, and Stat5 (27–29). In this study we were able to demonstrate that Pyk2 alone was able to activate Stat5, and this ability was dependent on its kinase activity and autophosphorylation site. Because tyrosine phosphorylation of Stats is required for activation (54–58), the association of FGFR3 and Pyk2 may play a role in increasing the proliferative and anti-apoptotic function of Stat5. Constitutive activity of Stat5 is associated with permanent changes in the expression of genes that inhibit apoptosis, increase proliferation, and induce angiogenesis (30, 59, 60). The increase in Stat5 activity caused by the interaction between FGFR3 and Pyk2, along with the known activation of the mitogen-activated protein kinase pathway by activated FGFR3, may be of underlying importance in the oncogenesis of malignancies such as multiple myeloma, where both FGFR3 and Pyk2 have already been implicated.

The results of this work are summarized in the model presented in Fig. 8. Signaling by Pyk2 is initiated by an autophosphorylation event at Tyr402. This phosphorylated residue then serves as a recruitment site for c-Src, which binds the Tyr(P)402 through one of its SH2 domains (19, 21). Subsequently, c-Src phosphorylates other tyrosine residues of Pyk2, and signaling

with anti-phospho-Stat5 (Tyr94) (Cell Signaling). The membrane was stripped and reprobed with anti-IGF (FL) to examine total GFP-Stat5B. The membrane was reprobed again with anti-Myc(9E10) to examine Pyk2 expression. The GFP-Stat3 expression was also determined. B, Pyk2 activation of GFP-Stat5B requires c-Src association, and activation is restored in the presence of activated FGFR3. 293T cells were transfected and immunoblotted as in A. C, quantification of activation of GFP-Stat5B. The tyrosine phosphorylation of GFP-Stat5B was normalized to total GFP-Stat5B expression using NIH Image software. The results shown are represented as percentages relative to myr-FGFR3-TD (100%). This bar graph represents the means of 3–9 independent experiments ± S.E.
Pyk2 requires an active kinase domain as well as the autophosphorylation site at Tyr402. FGFR3 has previously been shown to activate Stat5 when Tyr402 of Pyk2 is mutated to Phe. Of significant importance is the ability of FGFR3 – proteins including Shc and Grb2 (15, 22) – to activate Stat5, an important transcriptional regulator that participates in the cellular control of proliferation and apoptosis. As demonstrated here, one significant effect of the interaction between FGFR3 and Pyk2 is manifest in the regulation of activation of Stat5, an important transcriptional regulator that mediates increased Pyk2 phosphorylation, leading to activation of proliferative and/or anti-apoptotic signaling pathways. Although the kinase activity of Pyk2 is required for Stat5 activation, it appears that the association of FGFR3 with Pyk2 renders Pyk2 signaling c-Src-independent. FGFR3 activation of Stat5 is reduced in the presence of Pyk2-K457A.

At the present time, we have no clear evidence to identify the kinase that mediates increased Pyk2 phosphorylation. The kinase activity of Pyk2 itself may play a role, however, because FGFR3 hyperactivation leads to activation of Stat5 in human neoplasias like multiple myeloma in which Pyk2 has already been implicated (25, 26).

In summary, our results suggest that one role of hyperactivation of FGFR3 in human neoplasia may be the recruitment of Pyk2 to stimulate specific tyrosine phosphorylation, leading to activation of proliferative and/or anti-apoptotic signaling pathways. FGFR3 both contribute to the increased tyrosine phosphorylation of Pyk2 that is observed in response to FGFR3 hyperactivation leads to little or no phosphorylation of a Pyk2-K457A kinase-dead mutant (Fig. 5). Similarly, the FGFR3 kinase domain itself must play a role, because greater Pyk2 phosphorylation is observed in response to hyperactivated FGFR3-TD than with either wild type or kinase-dead FGFR3. Thus, it appears that the kinase domains of Pyk2 and FGFR3 both contribute to the increased tyrosine phosphorylation of Pyk2 that is observed in response to FGFR3 hyperactivation, although this may be indirect by recruitment of some other kinase (unlikely to be c-Src) to activated FGFR3 signaling complexes.

In summary, our results suggest that one role of hyperactivation of FGFR3 in human neoplasia may be the recruitment of FGFR3 to activate Stat5 alone (27). Here we show that Pyk2 also increases Stat5 activation measured by tyrosine phosphorylation. As presented in Fig. 8, the ability of FGFR3 to restore the activation of Stat5 in the presence of the Pyk2-Y402F mutant is of significant importance. Although the kinase activity of Pyk2 is required for Stat5 activation, it appears that the association of FGFR3 with Pyk2 renders Pyk2 signaling c-Src-independent. Being that c-Src is a major regulator of Pyk2 activation and signaling (19, 21), the association of FGFR3 with Pyk2 circumvents this activation and may cause aberrant signaling by Pyk2 that in turn could play a role in neoplasias like multiple myeloma in which Pyk2 has already been implicated (25, 26).

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Fig. 8. Model of Pyk2 and FGFR3 activation of Stat5. Left panel, to activate Stat5, Pyk2 requires an intact kinase domain as well as the autophosphorylation site at Tyr402. Middle panel, mutations within the kinase domain (kinase-dead Pyk2-K457A) or at Tyr402 (Pyk2-Y402F) prevent this activation of Stat5 by Pyk2. FGFR3 has previously been shown to activate Stat5. Of significant importance is the ability of FGFR3 to restore the activation of Stat5 when Tyr402 of Pyk2 is mutated to Phe. Right panel, although the kinase activity of Pyk2 is required for Stat5 activation, it appears that the association of FGFR3 with Pyk2 renders Pyk2 signaling c-Src-independent. FGFR3 activation of Stat5 is reduced in the presence of Pyk2-K457A.

proceeds through binding of other SH2 domain-containing proteins including Shc and Grb2 (15, 22–24). To activate Stat5, Pyk2 requires an active kinase domain as well as the autophosphorylation site at Tyr402.
