Cross-talk between Phospho-STAT3 and PLCγ1 Plays a Critical Role in Colorectal Tumorigenesis

Peng Zhang, Yiqing Zhao, Xiaofeng Zhu, David Sedwick, Xiaodong Zhang, and Zhenghe Wang

Abstract

Hyperphosphorylation at the Y705 residue of signal transducer and activator of transcription 3 (STAT3) is implicated in tumorigenesis of leukemia and some solid tumors. However, its role in the development of colorectal cancer is not well defined. To rigorously test the impact of this phosphorylation on colorectal tumorigenesis, we engineered a STAT3 Y705F knock-in to interrupt STAT3 activity in HCT116 and RKO colorectal cancer cells. These STAT3 Y705F mutant cells fail to respond to cytokine stimulation and grow slower than parental cells. These mutant cells are also greatly diminished in their abilities to form colonies in culture, to exhibit anchorage-independent growth in soft agar, and to grow as xenografts in nude mice. These observations strongly support the premise that STAT3 Y705 phosphorylation is crucial in colorectal tumorigenesis. Although it is generally believed that STAT3 functions as a transcription factor, recent studies indicate that transcription-independent functions of STAT3 also play an important role in tumorigenesis. We show here that wild-type STAT3, but not STAT3 Y705F mutant protein, associates with phospholipase Cγ1 (PLCγ1). PLCγ1 is a central signal transducer of growth factor and cytokine signaling pathways that are involved in tumorigenesis. In STAT3 Y705F mutant colorectal cancer cells, PLCγ1 activity is reduced. Moreover, overexpression of a constitutively active form of PLCγ1 rescues the transformation defect of STAT3 Y705F mutant cells. In aggregate, our study identifies previously unknown cross-talk between STAT3 and the PLCγ1 signaling pathways that may play a critical role in colorectal tumorigenesis.

Introduction

Signal transducer and activator of transcription 3 (STAT3) is thought to be an oncogene (1). Several lines of evidence support such a premise. First, persistent STAT3 activation has been detected in leukemia and in a variety of solid tumors including breast, brain, pancreas, ovarian, and squamous cell carcinomas of head and neck (SCCHN) cancers, and melanomas (2). Second, constitutively active STAT3 transforms rat and mouse cells and dominant negative STAT3 blocks Src-induced transformation in vitro (3, 4). Interestingly, recent studies show that the mitochondrial functions of STAT3 may be important factors in tumorigenesis, because its mitochondrial activity appears to be required for Ras-mediated tumor transformation (5, 6). Third, transgenic mice with keratinocytes expressing constitutively active STAT3 develop hyperproliferative dermatologic disorders in vivo (7). Fourth, targeted deletion of STAT3 in skin cells prevents epithelial cancers in mice (8), and targeting STAT3 specifically in B and T cells prevents development of lymphomas and myelomas (9). Latent cytoplasmic STAT3 becomes activated through phosphorylation of Y705 by cytoplasmic nonreceptor tyrosine kinases including Janus-activated kinase (JAK) and Src (10). Phosphorylated STAT3 dimerizes through reciprocal Src Homology 2 (SH2)-phosphotyrosine interaction and accumulates in the nucleus (2). Therefore, STAT3 activates the transcription of a wide array of genes including B-cell lymphoma-extra large (Bcl-XL) and suppressor of cytokine signaling 3 (SOCS3; ref. 2). Although the kinases that phosphorylate the Y705 residue of STAT3 are well defined in epithelial and hematopoietic cells, the phosphatases that specifically dephosphorylate pY705 have received little attention.

We previously identified STAT3 as a direct substrate of protein tyrosine phosphatase receptor T (PTPRT; ref. 11). PTPRT is mutated in colon, lung, stomach, and skin (melanoma) cancers (12). Moreover, PTPRT knockout mice are highly susceptible to azoxymethane-induced colon tumors (13), indicating that PTPRT normally functions as a tumor suppressor. Our finding that PTPRT specifically

Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

Corresponding Authors: Zhenghe Wang, Case Western Reserve University, 10900 Euclid Avenue, WRB 3120, Cleveland, OH 44106-7283, Phone: 216-368-0446; Fax: 216-368-8819; E-mail: zhenghe.wang@case.edu or Xiaodong Zhang, College of Life Sciences, Wuhan University, Wuhan, China; Phone/Fax: 86-27-68756606; E-mail: zhangxd@whu.edu.cn

doi: 10.1158/1541-7786.MCR-11-0147

©2011 American Association for Cancer Research.
dephosphorylates STAT3 at the Y705 residue supports a critical role for regulation of STAT3 Y705 phosphorylation in colorectal tumorigenesis. Although STAT3 is implicated in oncogenesis of leukemia, skin, and head and neck cancers (1), the impact of STAT3 Y705 phosphorylation in colorectal tumorigenesis has not heretofore been well defined. Here we show that successful knock-in (KI) of the STAT3 Y705F mutant allele into 2 different colorectal cancer cell lines results in mutant colorectal cancer cells that are less tumorigenic both in vitro and in vivo. The results of this study further suggest that modulation of tumorigenicity is at least partially dependent on STAT3 cross-talk with phospholipase Cγ1 (PLCγ1) through effects on S1248 phosphorylation. PLCγ1 is a key signaling molecule that hydrolyzes phosphatidylinositol-4,5-bisphosphate to generate inositol-1,4,5-triophosphate (IP3) and 1,2-diacylglycerol (DAG), which, in turn, activate intracellular Ca2+ and protein kinase C (PKC) signaling pathways that are implicated in tumorigenesis (14). In support, we show that colorectal cancer cells carrying STAT3 mutated in Y705 also exhibit reduced PKC activities.

Materials and Methods

Cell culture
HCT116, RKO, and HEK 293T cells were obtained from the American Type Culture Collection. HCT116 and RKO colorectal cancer cells were maintained in McCoy 5A media plus 10% FBS. HEK 293T cells were maintained in Dulbecco's modified Eagle's medium plus 10% FBS.

Somatic cell gene targeting
Somatic cell gene targeting was conducted as described (15, 16). Briefly, a 1.3-kb fragment from intron 21 to intron 22 of the STAT3 locus containing the exon 22 sequences was amplified 25 cycles from genomic DNA, using primers 5'-TAGCACCCTAGTCCGTTACCTGATATCGGAGAGTCACAG-3' and 5'-TGACCACCAGCGGAGGCTTCATTCTTATCCCACCTTA-3'. The coding sequences for Y705 were then mutated from TAC (Tyr) to TTC (Phe) by site-directed mutagenesis, using primers 5'-TCGCAGGCCTGCCCATTCCGTGAAGCAAGATTTTATC-3' and 5'-AATGGGCGACCCGGCCTGGA-3'. This mutated fragment was used as the right homologous arm and cloned in to an pAAV-Neo-Lox P vector with sodium orthovanadate, 50 mmol/L NaF, 80 mmol/L sodium chloride, 1 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 50 mmol/L NaF, 80 mmol/L β-glycerophosphate, and 20 mmol/L sodium pyrophosphate. Western blots were then sequenced to ensure the presence of the mutant Y705F alleles. To target the second allele with the same targeting vector, correctly targeted clones were infected with adenoviruses expressing the Cre-recombinase to delete the drug selection marker. To select clones with successful deletion of the drug selection marker, 30 cycles of genomic PCR were carried out to amplify an approximately 200-bp genomic fragment in which the Lox P site was inserted (using primers 5'-GCAGATGGAGCTTTCAGAC-3' and 5'-CGCCTGGAAGAGAAC-3'). The heterozygous KI clones were infected with the same targeting virus to target the second allele and the neomycin resistance gene was excised as described earlier.

Plasmid transfection
Cells were plated 1 day prior to transfection to achieve a 70% confluence at the time of transfection. Plasmids were transfected with the Lipofectamine Transfection Reagent (Invitrogen; catalogue no. 18324-020) according to the manufacturer's instructions. To transfect a T75 flask of HEK 293 cells, 9 μg of plasmids were mixed with 54 μL of Lipofectamine Transfection Reagent and 1.5 mL of OptiMEM (Invitrogen; catalogue no. 31985070). The transfection mixture was then incubated with cells at 37°C for 4 hours. Cells were subsequently cultured in normal medium after washing once with Hank's balanced salt solution.

Western blot
Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer with complete protease inhibitor mixture and phosphatase inhibitors [50 mmol/L Tris-HCl (pH 8.0), 0.5% triton X-100, 0.25% sodium deoxycholate, 150 mmol/L sodium chloride, 1 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 50 mmol/L NaF, 80 μmol/L β-glycerophosphate, and 20 mmol/L sodium pyrophosphate]. Western blot analysis was performed using antibodies (Table S1). Antibodies used included anti-pY705 (Cell Signaling Technology), and the antibodies listed in the Supplementary Table S1.

Immunofluorescence staining
Cells were seeded on glass cover slips and grown to 50% confluence and serum starved for 18 hours. A subset of cells was treated with 10 ng/mL of interleukin-6 (IL-6) for 30 minutes following by fixation with 4% paraformaldehyde.
for 30 minutes at room temperature. The fixed cells were permeabilized with 0.2% Triton X-100 at room temperature for 5 minutes and then blocked with Image-iT FX signal enhancer (Invitrogen) at room temperature for 30 minutes. Immunofluorescent staining was done with anti-STAT3 antibody (Santa Cruz Biotechnology) and the Alexa 488–conjugated anti-rabbit secondary antibody (Invitrogen). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; 1 μg/mL) at room temperature for 20 minutes. Images were captured with a LSM 510 META confocal microscope (Carl Zeiss MicroImaging).

Flow cytometry

Cell were harvested during the log phase of growth and fixed with methanol. Cells were then incubated at 37°C for 30 minutes in 5% normal goat serum diluted in PBS. Propidium iodide (PI) solution (100 μg/mL; 0.1% NP40; 0.1% sodium azide) was used to stain cells at 4°C for 1 hour. Cells were analyzed on an Epics XL flow cytometer (Beckman Coulter). WinMDI2.9 was used for data analysis. Cell debris and aggregates were excluded on PI gating. Percentages of G₁, S, and G₂–M populations were determined by histograms generated by WinMDI2.9.

Reverse transcriptase-PCR

Total RNAs were isolated from 1 million cells by using the Qiagen RNAeasy Mini Kit according to the manufacturer’s instructions and cDNAs were synthesized by the SuperScript III First-Strand Synthesis System (Invitrogen). The 5'TCCCAGAAAGGATACAGCTGG-3' and 5'ACTGAAAGATGACCCCCAGCAG-3' primers were used to PCR amplify Bcl-XL. The 5'GGATCTGG-TACCTGCTCTTGGA-3' and 5'AGGCTCCTTTTGT-GACCTTCA-3' primers were used to amplify SOCS3. The PCR products were resolved on 1% agarose gel. The PCR mixtures were prepared according to the manufacturer’s instructions; the PCR mixtures were run on a GeneAmp PCR system 9700 (Applied Biosystems) at 94°C for 30 seconds, 55°C for 30 seconds, 70°C for 30 seconds for 25 cycles.

Colony formation assay

HCT116 and RKO cells were trypsinized, counted twice by using a hemocytometer, and placed into 6-well plates at 400 cells per well. Cells were grown for 14 days before staining with Crystal Violet (Sigma). The experiment was repeated 3 times with 2 replicates each. Average numbers of colonies from each experiment were plotted.

Focus formation assay in soft agar

HCT116 and RKO clones were trypsinized, counted twice by a hemocytometer, and plated at 5,000 cells/mL in top plugs consisting of 0.4% SeaPlaque agarose (FMC Bioproducts) and McCoy’s 5A medium. After 30 days, the colonies were photographed and counted. The experiment was repeated 3 times with 2 replicates each. Average numbers of colonies from each experiment were plotted.

Boyden chamber cell migration and invasion assay

Cell migration and invasion assay was carried out as previously described (18). Transwell membranes (pore size 8.0 μm; Corning Inc.) were coated with either 50 μg/mL fibronectin, 12.5 μg/mL of collagen IV or 1 mg/mL of Matrigel. The matrices were coated on membranes according to the manufacturer’s instructions. Specifically, Matrigel (BD Biosciences) was thawed overnight at 4°C and then diluted in serum-free medium to 1 mg/mL, and 100 μL of the diluted Matrigel was added to upper chamber of 24-well and incubated at 37°C for 2 hours. Cells were detached with 2 mmol/L EDTA and resuspended in serum-free medium with 0.1% of bovine serum albumin. Five hundred thousand cells were added to the upper compartment of the transwell chamber in the wells of a 24-well plate and allowed to migrate to the underside of the inserts for 24 hours. Complete medium containing 10% FBS was added in the lower compartment as a chemoattractant. Nonmigrating cells on the upper membrane were removed with a cotton swab, and cells that had migrated and become attached to the bottom surface of the membrane were fixed and stained with crystal violet. Migrated cells were counted microscopically at 200× magnification. Five randomly chosen fields were counted for each transwell membrane. The experiments were repeated 3 times with 2 replicates for each cell line. Average numbers of migrated cells per field from each experiment were plotted.

Xenograft

Five million cells were injected subcutaneously and bilaterally into 4- to 6-week-old female nude mice (5 nude mice in each group). Tumor formation and size were assessed by weekly caliper measurements of the length and width of the tumors. Tumor volumes were calculated by the formula: Volume = (width)² × length/2. After 21 days, the mice were sacrificed and tumors were harvested.

Construction of PLCγ1-myc tag and PLCγ1 mutant plasmid

To facilitate molecular cloning, we constructed a pCMV-USER-3xMyc vector by inserting a USER cassette into the pCMV-3Tag-2A plasmid (Agilent Technologies). Briefly, primers 5'-AATTCGATATCGCTGGAGTGCCCCATCTGA- GAGATCTCTAGACTATGCGCTACGC-3' and 5'-TGAGCTGGAGGCTAGCTCTAGAGGATCCTCTAG-0 were annealed together and cloned into the pCMV-3Tag-2A plasmid, using EcoRI and XhoI restriction enzymes. The open reading frame of PLCγ1 was PCR amplified from a human PLCγ1 cDNA purchased from Open Biosystems, using primers 5'-GGTCCCA(d)Utggcgggcgtcgcgaccccct-3' and 5'-GGCATAG(d)Uttaaagagagcacttccaca-3' and then cloned into the pCMV-USER-3xMyc vector by using the USER cloning system (New England Biolabs) according to the manufacturer’s instructions. The construct was sequenced to ensure no mutation was introduced by PCR. Constitutively active PLCγ1 Y509A, F510A, and D1019L mutations were then introduced into the
Myc-tagged PLCγ1 plasmid by 2-step site-directed mutagenesis. The first step created the PLCγ1 Y509A and F510A double mutant, using primers 5'-TGACCAACATGGGCAACACTCTCAACA-3' and 5'-TGACCAAGATCTGCTGCTAGTCAAGGATACCAC-3'. The second step generated the D1019L mutation in the above double-mutant plasmid, using primers 5'-GGGTCATAATGGGAGAGTAGGCTGGGCTGGCTATTAGCCCT-3' and 5'-CTCTGCTCCAATTATGACCT-3'. The details of site-directed mutagenesis methods are described in ref. 19.

Glutathione S-transferase–fusion protein pull-down

Sequences encoding the 2 tandem SH2 domains of PLCγ1 were amplified by PCR from PLCγ1 cDNA, using primers 5'-TGACCAGAATTCGAGAAGTGGTTCCAGGGAAG-3' and 5'-TGGTCAGTCAAGGATCAGGGGCTTCTCA-3'. The PCR product was then cloned into pGEM-6p-1 (GE Healthcare), using EcoRI and XhoI restriction enzymes. Recombinant proteins were expressed and purified from Escherichia coli. Twenty million HCT116 cells were lysed in RIPA buffer with complete protease inhibitor mixture and phosphatase inhibitors (see Western blot section for the recipe) for STAT3 pull-down. GST–PLCγ1–SH2 domain fusion protein (1 μg) bound beads were incubated with the cell lysate at 4°C for 1 hour. The beads were washed and then boiled and the aliquots were analyzed by SDS-PAGE and Western blotting. Equal amount of glutathione S-transferase (GST) beads were treated identically as a control.

Statistical analyses

All statistical analyses were carried out by the SAS software (SAS Institute). We applied the t test to compare the means between 2 groups assuming unequal variances. For xenograft growth, we carried out MANOVA for repeated measurements to test whether there is an overall difference in the tumor sizes by testing group difference as well as whether there was a difference in development of tumor sizes over time between the 2 groups by testing the interaction between time and group.

Results

Engineering STAT3 Y705F knock-in colorectal cancer cell lines

To rigorously test whether regulation of STAT3 Y705 phosphorylation is critical to colorectal tumorigenesis, we set out to engineer STAT3 Y705F KI colorectal cancer cell lines. The AAV targeting system was used to engineer the KI cell lines because of its high homologous recombination frequency in somatic cells (20, 21). We first chose to knock in the STAT3 Y705F mutant allele into the human colon cancer cell line HCT116, because we had shown that STAT3 can be activated by IL-6 in HCT116 cells (11) and because HCT116 had been widely used for successful

---

**Figure 1.** Engineering Y705F STAT3 KI colorectal cancer cell lines. A, diagram of the KI construct. B and C, PCR products of the KI clones after excision of the neomycin resistance gene by Cre-recombinase. P indicates parental cells; WT/KI indicates Y705F STAT3 heterozygous KI cells; KI/KI indicates Y705F STAT3 homozygous KI cells; arrow indicates the KI allele. D and E, parental, Y705F heterozygous and Y705F homozygous cells were starved for 18 hours and treated with or without IL-6 for 30 minutes. Western blots were carried out with pSTAT3 or STAT3 antibodies.
gene targeting by homologous recombination (15, 20). The targeting strategy is outlined in the schematic diagram in Figure 1A. After the first round of gene targeting, 4 targeted clones were identified out of 192 geneticin resistant clones screened. To ensure the presence of the mutant allele, STAT3 exon 22 genomic PCR products of the targeted clones were DNA sequenced. Three of the 4 targeted clones harbored STAT3 Y705F mutant allele (Supplementary Fig. S1). Two clones were infected with adenovirus expressing Cre-recombinase to excise the neomycin resistance gene (Fig. 1B) and targeted for the second allele to generate homozygous KI clones. To confirm that there was no wild-type (WT) STAT3 allele expression in the homozygous Y705F STAT3 clones, the RT-PCR products of the targeted clones were DNA sequenced. Three of the 4 targeted clones harbored STAT3 Y705F mutant allele (Supplementary Fig. S1).

STAT3 Y705F mutant failed to activate its target genes

Our previous studies showed that IL-6 induces STAT3 translocation from the cytoplasm to the nucleus and activates transcription of its target genes, Bcl-XL and SOCS3 in colorectal cancer cells (11). To delineate the role of STAT3 phosphorylation in the translocation process, current experiments examined whether IL-6 induces STAT3 Y705F mutant protein translocation. This translocation process was directly monitored in parental and homozygous STAT3 Y705F mutant HCT116 and RKO cells using immunofluorescence staining. As shown in Figure 2A and Supplementary Figure S2A, STAT3 Y705F mutant proteins remain diffused on IL-6 stimulation, whereas WT STAT3 proteins accumulate in the nucleus in parental cells. Then, we utilized gene expression analyses by RT-PCR to show that IL-6 fails to activate gene transcription of Bcl-XL and SOCS3, 2 of the STAT3 target genes, in STAT3 Y705F homozygous KI cells (Fig. 2B; Supplementary Fig. S2B). Taken together, these data showed that STAT3 Y705F mutant is unresponsive to IL-6 stimulation, and strongly suggest that STAT3 Y705 phosphorylation is critical for its activation in colorectal cancer cells.

STAT3 Y705F mutant colorectal cancer cells grew slower than parental cells in tissue culture

When grown under normal tissue culture conditions (McCoy's 5A supplemented with 10% FBS) over a 4-day period, both HCT116 and RKO STAT3 Y705F homozygous KI cells grew slower than their parental cells (Fig. 3A). Although the doubling times of the parental HCT116 and STAT3 Y705F mutant cells were not significantly different (Supplementary Fig. S3A), the average doubling times of the RKO STAT3 Y705F mutant clones increased by 4 hours in comparison with the parental cells (Supplementary Fig. S3A). Consistently, compared with WT cells, the STAT3 Y705 mutant RKO cells had an elevated G1 population, whereas no cell cycle profile difference was observed among the HCT116 parental and mutant clones (Supplementary Fig. S3B). Given that STAT3 is also involved in tumor invasion and metastasis (22), we set out to determine whether the STAT3 Y705 mutation affects colorectal cancer cell migration and invasion. As shown in Supplementary Figure S4, no significant difference was found between the parental and the homozygous KI cells on fibronectin, collagen IV, and Matrigel matrices.
In vitro, STAT3 Y705F mutant colorectal cancer cells are reduced in properties predictive of in vivo tumorigenicity

To test whether STAT3 Y705F mutant affects tumorigenicity-correlated responses in vitro, we carried out colony formation and soft agar assays with the STAT3 mutant KI cells. Compared with the parental cells, homozygous STAT3 Y705F KI HCT116 and RKO cells exhibited 3- to 5-fold ($P < 0.001$) reduced abilities to form colonies in colony-formation assay (Fig. 3B). Similarly, homozygous STAT3 mutant colorectal cancer cell clones formed approximately 3-fold ($P < 0.001$) less foci in soft agar assay than their WT counterparts (Fig. 3C). Interestingly, all of the heterozygous KI clones also displayed significant ($P < 0.05$) reduction in colony numbers and soft agar foci with respect to WT cells (Fig. 3B and C).

STAT3 Y705F mutant colorectal cancer cells were less tumorigenic in vivo

Tumorigenicity of the KI cells was also tested in a more stringent in vivo model. For these studies, STAT3 Y705F homozygous, heterozygous clones or the parental HCT116 and RKO cells were injected subcutaneously into nude mice. Tumor formation and size were assessed by weekly caliper measurements. After 21 days of growth, WT cells formed tumors in all mice injected, whereas each of the STAT3 Y705F homozygous KI clones failed to form tumors in at least 1 of the 5 mice injected (Fig. 4A). The average tumor volumes of STAT3 Y705F homozygous KI clones were 10-fold smaller than those produced by the parental cells for both the HCT116 ($P < 0.001$) and RKO ($P < 0.0001$) colorectal cancer cell lines (Fig. 4B). Furthermore, the development of tumor sizes over time for STAT3 Y705F homozygous KI clones was significantly slower than

![Figure 3. STAT3 Y705F mutant colorectal cancer cells are less tumorigenic in vitro.](image-url)
that of the parental cells for both the HCT116 ($P < 0.001$) and RKO ($P < 0.0001$). Notably, the average tumor sizes of STAT3 Y705F heterozygous KI clones were also significantly ($P < 0.05$) less than those of parental cells (Fig. 4B).

**STAT3-modulated PLCγ1 activity**

The studies above showed that phosphorylation of the STAT3 Y705 residue plays a critical role in colorectal tumorigenesis. It was also desirable to gain insights into the effects of this phosphorylation on downstream signaling. In these studies, we examined how the STAT3 Y705F KI affects phosphorylation of other signaling molecules in colorectal cancer cells after IL-6 stimulation. It is well documented that IL-6 activates multiple well-characterized signaling pathways including Ras-MAPK, PI3K-AKT, and PLCγ (23). Therefore, we tested the phosphorylation status of 26 sites on 17 proteins on the basis of their ability to respond to IL-6 stimulation (Supplementary Table S1). Among these candidates, PLCγ S1248 phosphorylation was consistently elevated in STAT3 Y705F mutant cells in comparison with the parental cells (Fig. 5A; Supplementary Fig. S5). As S1248 phosphorylation negatively regulates PLCγ1 activity (24), this result suggests that PLCγ1 is less active in STAT3 Y705F mutant cells. In support, pPKC levels, an immediately downstream target of PLCγ1, were also reduced in the STAT3 Y705F mutant cells (Fig. 5A; Supplementary Fig. S5).

We speculated that STAT3 might physically interact with PLCγ1 leading to modulation of its activity. In support, STAT3 was shown to form complexes with PLCγ1 under both overexpression and physiologic conditions, as shown by reciprocal immunoprecipitations (Fig. 5B and C). Interestingly, the WT, but not the STAT3, Y705F mutant proteins interact with PLCγ1. Furthermore, the STAT3 proteins immunoprecipitated by PLCγ1 were phosphorylated (Fig. 5C). These results suggest that STAT3 phosphorylation may play a role in its physical interaction with PLCγ1. In support, the STAT3-PLCγ1 interaction was readily detected when cells were stimulated with IL-6 (Fig. 5D), but was barely detectable under starvation conditions. Moreover, this interaction is mediated by the 2 SH2 domains of PLCγ1, as recombinant PLCγ1 SH2 domains pulled STAT3 down from HCT116 lysates (Fig. 5E).

Constitutively active PLCγ1 rescued the colony formation defect of STAT3 Y705F mutant cells

The functional significance of the cross-talk between STAT3 and PLCγ1 was then tested by determining whether constitutively active PLCγ1 mutant could phenotypically rescue STAT3 Y705 mutant cells. A PLCγ1 triple mutant
construct (D1019L, Y509A, and F510A), which is well documented to be constitutively active (25), was introduced into STAT3 Y705F mutant HCT116 cells. As shown in Figure 5F, the mutant PLCγ1 partially rescued the colony formation defect of the STAT3 Y705F mutant cells, suggesting that PLCγ1 is a critical downstream mediator of STAT3 oncogenic signaling.

Discussion

Using genetically engineered STAT3 Y705 KI colorectal cancer cells, we show that STAT3 phosphorylation can regulate the efficiency of colorectal tumorigenesis. Unexpectedly, extension of studies of the STAT3 Y705F mutant also shows that cross-talk between STAT3 and PLCγ1 signaling pathways may provide further mediating mechanisms that modulate colorectal tumorigenesis.

PLCγ1 is a major signal transducer of growth factor and cytokine signaling (26). PLC hydrolyzes phosphatidylinositol-4,5-biphosphate to generate IP3 and DAG. Although IP3 modulates intracellular Ca2+ signaling, DAG activates PKCs (26). Results of our studies show, for the first time, that STAT3 modulates PLCγ1 activity. In this regard, decreased PKC activities in STAT3 Y705F mutant colorectal cancer cells suggest that STAT3 activates PLCγ1 (Fig. 5A; Supplementary Fig. S5). However, it remains to be

Figure 5. STAT3 cross-talks with PLCγ1. A, parental and STAT3 Y705F KI cells were stimulated with IL-6 for the indicated time. Cell lysates were blotted with the indicated antibodies. See Supplementary Figure S5 for quantification. B, HEK 293 cells were transfected with the indicated plasmids. Cell lysates were immunoprecipitated with either anti-Flag or anti-Myc antibodies and blotted with the indicated antibodies. Arrow indicates co-immunoprecipitated Flag-STAT3. C, cell lysates from parental and STAT3 Y705F KI cells were immunoprecipitated with antibodies against either STAT3 or PLCγ1 and blotted with the indicated antibodies. D, HCT116 cells were serum-starved for 16 hours and then stimulated with or without 10 ng/mL of IL-6 for 30 minutes. Cells were then lysed and immunoprecipitated with either anti-PLCγ1 antibodies or control immunoglobulin G (IgG). Immunocomplexes were resolved on 8% SDS-PAGE and Western blots were carried out with the indicated antibodies. E, HCT116 cell lysates were incubated with 1 μg of either GST–PLCγ1–SH2 domains or GST alone for 1 hour at 4°C. The pull-down mixtures were resolved on SDS-PAGE and blotted with an anti-STAT3 antibody. F, STAT3 Y705F KI cells were transfected with either an empty vector or a vector expressing PLCγ1 triple mutant (D1019L, Y509A, and F510A). Colony formation assay was performed. *, P < 0.05; **, P < 0.001, t-test. Western blot was carried out to show expression of myc-tagged PLCγ1 mutant protein.
determined whether STAT3 can regulate intracellular Ca$^{2+}$ signaling. Furthermore, our data suggest that activation of PLCγ1 by STAT3 requires physical interaction between the 2 proteins and that this interaction seems to be mediated by STAT3 pY705 and the SH2 domains on PLCγ1.

Given that numerous studies suggest that PLCγ1 is involved in tumor progression (14), it is not surprising that overexpression of constitutively active PLCγ1 can partially rescue the colony formation defect of STAT3 Y705F KI colorectal cancer cells. Data presented here strongly suggest that a transcription-independent function of STAT3 is at least partially involved in critical control of tumor transformation and that the general belief that STAT3 mainly functions through its transcriptional activity is an oversimplification. In this regard, recent studies show that the mitochondrial functions of STAT3 may be important factors in tumorigenesis, because its mitochondrial activity appears to be required for Ras-mediated tumor transformation (5, 6). Notably, however, our studies show that PLCγ1 only partially rescues the defect in the STAT3 Y705F mutant cells suggesting that STAT3 transcriptional activity plays an important role in regulating STAT3's oncogenic functions.

Our data indicate that STAT3 Y705F mutant proteins fail to accumulate in the nucleus on IL-6 stimulation (Fig. 2; Supplementary Fig. S2). However, the unphosphorylated STAT3 proteins can still be imported into the nucleus, as shown by the observation that a significant portion of STAT3 Y705F mutant protein is localized in the nucleus in both HCT116 and RKO colorectal cancer cells (Fig. 2). This observation is consistent with a previous study showing that STAT3 nuclear importation is independent of tyrosine phosphorylation and that the N-terminal coiled-coil domain of STAT3 is important for its nuclear importation (27). In fact, numerous studies show that STAT3 proteins translocate and accumulate in the nucleus after cytokine and growth factor stimulation (1). In this regard, the data from our laboratory show unequivocally that the nuclear accumulation of STAT3 is dependent on STAT3 Y705 phosphorylation. Although we showed that STAT3 Y705F mutant proteins failed to activate IL-6–induced target genes, Bel-7402 and SOCS3, our data do not exclude the possibility that the mutant STAT3 can activate transcription of other non-canonic STAT3 target genes. Interestingly, recent studies showed that overexpression of the STAT3 Y705F mutant induces expression of genes that are distinct from those induced by pSTAT3 (28).

STAT3 activity is upregulated in various cancers including colorectal cancers (2). Although STAT3 has not yet been found to be mutated in human cancers, kinases and phosphatases that regulate STAT3 Y705 phosphorylation are mutated in a variety of cancers that result in STAT3 activation. Activating mutations of JAK2 are found in the majority of myeloproliferative neoplasms (29), whereas inactivating mutations of phosphatases PTPRT and PTPRD that dephosphorylate STAT3 are found in various solid tumors (12, 30, 31). Although our previous study showed that STAT3 is a direct substrate of PTPRT that is mutated in colorectal cancers (11), this study clearly shows that regulation of STAT3 Y705 phosphorylation plays a critical role in colorectal tumorigenesis, because STAT3 Y705F homozygous KI colorectal cancer cells grow slower than the parental cells both in tissue culture and in tumor xenograft models. Interestingly, STAT3 Y705F heterozygous KI cells also exhibit reduced tumorigenicity, suggesting that STAT3 Y705F protein may act as either a dominant negative or haploid-insufficiency mutant. Although our data cannot distinguish these 2 possibilities, several previous studies suggest that STAT3 Y705F mutant has a dominant negative effect (4, 32, 33).

Finally, our data indicate that inactivation of STAT3 in colorectal cancer cells slows down tumor growth both in tissue culture and in tumor xenograft models (Figs. 3A and 4). Interestingly, RKO STAT3 Y705F KI cells progress slower through cell cycle, but no cell cycle defect was observed in HCT116 STAT3 Y705F mutant cells. It is possible that HCT116 STAT3 mutant cells grow slower through other mechanisms (e.g., The cells may be less responsive to growth factor stimulation). Our study suggests that the STAT3 signaling pathway may be a good target for colorectal cancer therapy. It is conceivable that interference with STAT3 phosphorylation, dimerization, and DNA binding processes or inhibition of its upstream kinase(s) could be exploited to inhibit the tumorigenic growth of colorectal cancers. Indeed, peptides, peptidomimetics and small chemical compounds that target STAT3 dimerization have shown promising inhibition of in vitro growth of breast cancer cells as well as cancer cells from other tissue types (34, 35, 36). Recently, a combination of in silico approaches and chemical screens led to identification of several small molecules that potently inhibit STAT3 activities and cause breast cancer cell death in both in vitro and in vivo models (37, 38). Further, platinum compounds that block the DNA binding of phosphorylated STAT3 were also found to inhibit cancer cell growth (39). Along the same line, Dr. Jennifer Grandis’ group has successfully used a decoy oligonucleotide that blocks the transcription activity of STAT3 to inhibit tumor growth of SCCCHN xenografts which harbor persistently active STAT3 (40, 41). Moreover, targeting STAT3 activation by inhibiting upstream kinases with chemical compounds also caused potent antitumor effect (42). Our data provide a strong rationale for exploring existing and emerging STAT3 inhibitors alone and in combination as targeted therapy for colorectal cancers. Furthermore, our STAT3 Y705F mutant clones and their parental cells should provide ideal reagents for testing the specificity of STAT3 inhibitory compounds that target STAT3 Y705 phosphorylation.

Disclosure of Potential Conflicts of Interests

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Drs. Sanford Markowitz and Chao Wang for helpful discussions and critical reading of this manuscript.
Grant Support

This research was supported by grants from the NIH (RO1-CA127590, RO1-HG00722, RO1-HG003054) and the V foundation.

References


Molecular Cancer Research

Cross-talk between Phospho-STAT3 and PLCγ1 Plays a Critical Role in Colorectal Tumorigenesis

Peng Zhang, Yiqing Zhao, Xiaofeng Zhu, et al.


Updated version

Access the most recent version of this article at:
doi:10.1158/1541-7786.MCR-11-0147

Supplementary Material

Access the most recent supplemental material at:
http://mcr.aacrjournals.org/content/suppl/2011/08/12/1541-7786.MCR-11-0147.DC1

Cited articles

This article cites 42 articles, 22 of which you can access for free at:
http://mcr.aacrjournals.org/content/9/10/1418.full#ref-list-1

Citing articles

This article has been cited by 2 HighWire-hosted articles. Access the articles at:
http://mcr.aacrjournals.org/content/9/10/1418.full#related-urls

E-mail alerts

Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions

To request permission to re-use all or part of this article, use this link
http://mcr.aacrjournals.org/content/9/10/1418.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.