

# Quantitative Phosphoproteomics Reveals a Cluster of Tyrosine Kinases That Mediates Src Invasive Activity in Advanced Colon Carcinoma Cells

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## Abstract

The nonreceptor tyrosine kinase Src is frequently overexpressed and/or activated in human colorectal carcinoma (CRC), and its increased activity has been associated with a poor clinical outcome. Src has been implicated in growth and invasion of these cancer cells by still not well-known mechanisms. Here, we addressed Src oncogenic signaling using quantitative phosphoproteomics. Src overexpression increased growth and invasiveness of metastatic SW620 CRC cells. Stable isotope labeling with amino acids in cell culture in combination with liquid chromatography tandem mass spectrometry allowed the identification of 136 proteins which exhibited a significant increase in and/or association with tyrosine phosphorylation upon Src expression. These mainly include signaling, cytoskeleton, and vesicular-associated proteins. Interestingly, Src also phosphorylated a cluster of tyrosine kinases, i.e., the receptors Met and EphA2, the cytoplasmic tyrosine kinase Fak, and pseudo-tyrosine kinase Sgk223, which were required for its invasive activity. Similar results were obtained with metastatic Colo205 CRC cells that exhibit high endogenous Src activity. We concluded that Src uses a tyrosine kinases network to promote its invasive activity in CRC and this implicates a reverse signaling via tyrosine kinase receptors. Targeting these tyrosine kinases may be of significant therapeutic value in this cancer. [Cancer Res 2009;69(6):2279–86]

## Introduction

The nonreceptor tyrosine kinase Src plays important roles in cell proliferation, survival, and migration induced by extracellular stimuli including growth factors and integrins (1). It also shows oncogenic activity when deregulated, a situation originally found with v-Src, the transforming product of the avian retrovirus Rous sarcoma virus (2). Since then, Src has been reported deregulated in human cancer (3). Remarkably, elevated kinase activity has been found in >80% of colon carcinoma (CRC) compared with the normal counterpart and this has been associated with a poor clinical outcome (3). How Src is deregulated in this cancer has not been clearly established yet. An activating mutation has been

reported in a minority of advanced tumors, suggesting the existence of alternative mechanisms for kinase deregulation (4). Accordingly, kinase activation often correlates with protein overexpression (2). This molecular event is however not sufficient in a normal cellular context as Src is subjected to strict regulation that keeps the enzyme in an inactive form. Therefore, it has been postulated that Src activation additionally implicates alteration of important Src regulators by genetic or epigenetic mechanisms, including the inhibitory COOH-terminal Src tyrosine kinase Csk (2).

A large body of evidence points to Src deregulation as an important event for colon tumorigenesis and metastasis (3). For instance, Src regulates growth, survival, and invasion of some CRC cell-lines *in vitro* (3, 5). Moreover, it contributes to tumor growth, angiogenesis, and metastasis in xenografts nude mouse models (3). Therefore, Src has become an attractive therapeutic target in this cancer and several small inhibitors are under clinical trials. Intriguingly, Src oncogenic signaling has been unraveled only partially. Although Src interacts with components of the focal contacts and the adherent junctions for cell migration (2), substrates important for cellular growth and invasiveness have been poorly described. Mass spectrometry (MS)-based quantitative phosphoproteomics has been a valuable tool to decipher signaling pathways initiated by a given tyrosine kinase (6). Among these, the stable isotope labeling with amino acids in cell culture (SILAC) has been used for the identification of oncogenic tyrosine kinase signaling such as HER2 (7) and Bcr-Abl (8). Here, we used this approach to address Src oncogenic signaling in advanced CRC cells. By overexpressing Src in SW620 cells, we identified 136 substrates and/or tyrosine phosphorylation-associated proteins. These include proteins with signaling, cytoskeleton, and vesicular trafficking functions, which may be important for the high Src invasiveness observed in these cells. Additionally, we identified a small cluster of tyrosine kinases that mediates this Src oncogenic signaling, uncovering a tyrosine kinases network important for the induction of cellular invasion. Targeting these tyrosine kinases may therefore be of significant value in advanced CRC therapy.

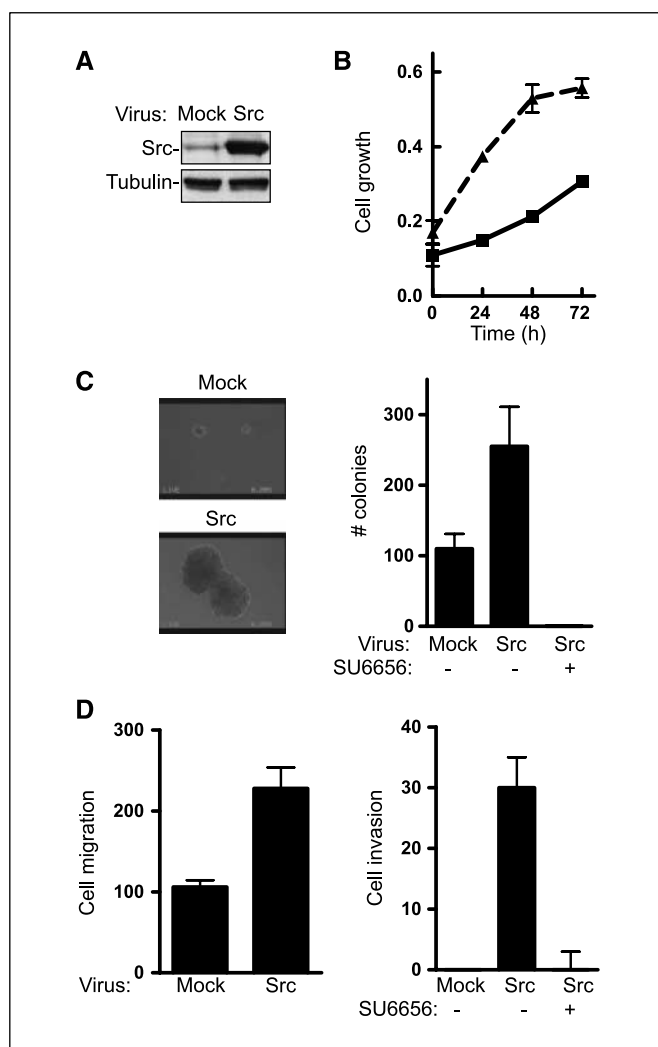
## Materials and Methods

**Reagents.** Human Src has been subcloned in pMX-pS-CESAR. Construct expressing Pragmin (a gift of Dr. Negishi, Kyoto University, Kyoto, Japan) and PragminY397F that was generated by mutagenesis have been subcloned into pBABE retroviral vectors. pRETRO-SUPER expressing shRNA used in this study were from TRANSAT. Targeting sequences inserted in shRNA constructs were GACACTCGGTAGTCTATAC (control), GATGAGT-TATTAGCAGAAG (Syk), GAAGTCTAACTATGAAGTA (Fak), GCAGTAT-ACGGAGCACTTC (EphA2), GTCACAGGCCAAGATAGAA (Sgk223), and TAAGAGCTGTGAGAATATA (Met). siRNA targeting sequence were form

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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**Figure 1.** Src promotes cell growth and invasiveness of SW620 cells. **A**, Western blot analysis showing the Src level in cells infected with control (mock) and Src expressing retroviruses as indicated. The level of tubulin is also shown. **B** and **C**, Src expression promotes growth of SW620 cells in standard medium and soft-agar conditions. **B**, time course of cell growth (arbitrary units) in standard conditions (square, mock-infected cells; triangle, src-infected cells). **C**, a representative example of colonies obtained with cells infected with control (mock) and Src expressing retroviruses in soft-agar medium at day 20 (left). Statistical analysis of number of colonies of cells infected with indicated retrovirus and treated with 5  $\mu$ mol/L SU6656 as shown (right). **D**, Src promotes cellular migration (left) and invasion (right) through Matrigel of SW620 cells *in vitro*. Number of cells/field that have migrated (C) or invaded through Matrigel (D) in Boyden chamber assays. Cells have been treated with 5  $\mu$ mol/L SU6656 when indicated. Columns, mean ( $n > 3$ ); bars, SD.

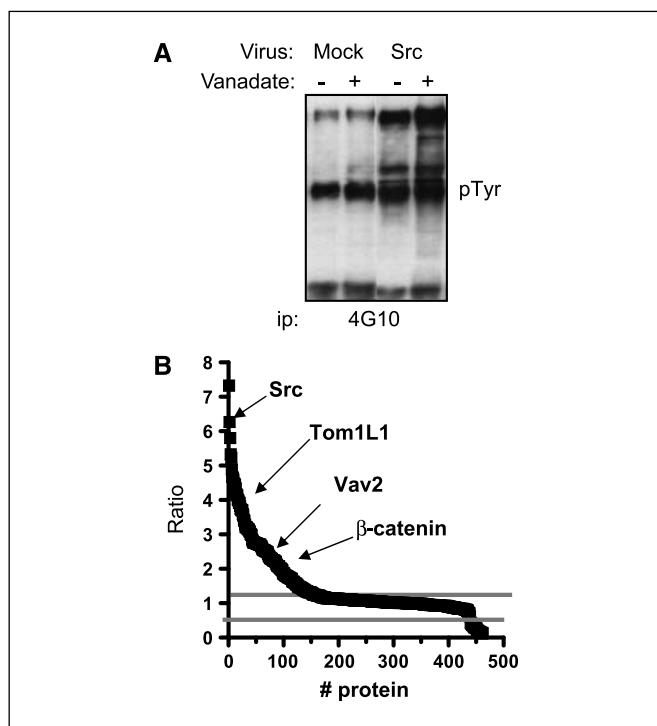
Dharmacon and included AATTCCTCGAACCTGTACAGT (control), AAAGATAACCTCTCATAATG (met), AATGACATGCCGATCTACATG (EphA2), and AAGCTAGTGACGTATGGATGT (Fak).  $^{13}\text{C}_6^{15}\text{N}_4$ -Arg and  $^{13}\text{C}_6^{15}\text{N}_2$ -Lys were from Invitrogen and Sigma Aldrich, respectively, anti-pTyr column from Upstate, Inc., SU6656 and PPI and PP2 from Calbiochem, and SU11274 and BAY 61-3606 from Sigma Aldrich. Antibodies used in this study were anti-4G10 (a gift from P. Mangeat, University of Montpellier, Montpellier, France), pY100 (CST), anti-ARAP3 (9), anti-Calponin (10), anti-p130Cas (Upstate, Inc.), anti- $\delta$ Catenin (BD Biosciences), anti-Cbl (sc-170; Santa Cruz Biotech), anti-CHC X22 (Alexis Biochemicals), anti-EphA2 (sc-924; Santa Cruz Biotech), anti-EphB2 (sc-28980; Santa Cruz Biotech), anti-ezrin (11), anti-Fak (Upstate, Inc.), anti-pY861Fak, (sc-16663; Santa Cruz Biotech), anti-Hrs A-5 (Alexis Biochemicals), anti-IRSp53 (12), anti-Met (sc-161; Santa Cruz Biotech), anti-pY1234/1235Met (Invitrogen), anti-Moesin was generated

against the purified protein as described by Andreoli and colleagues (11), anti-Odin (13), anti-Radixin was generated against the purified protein as described by Andreoli and colleagues (11), anti-Sam68 (14), anti-SgK223 (15) was raised against the GST-Pragmin NH<sub>2</sub> terminus, anti-SgK269 3G6 (Abnova GmbH), anti-SHIP1 (16), anti-SHP2 (sc-280; Santa Cruz Biotech), anti-Src family kinases (17), anti-p85 $\alpha$  subunit of PI3K (18), anti-PLC $\gamma$  (19), anti-pY418Src (Biosource), anti-Stam1/2 (20), anti-Syk (sc-1240; Santa Cruz Biotech), anti-pY325Syk and anti-pY524/525Syk (CST), anti-tubulin (a gift from N. Morin of CRBM, Montpellier, France), anti-Vav2 (sc-20803; Santa Cruz Biotech), anti-Wave2 (12), anti-ZO1 (sc-10804; Santa Cruz Biotech), and anti-ZO2 (sc-11448; Santa Cruz Biotech).

**Cell infections, growth, migration, and invasion.** SW620 and Colo205 cells (American Type Culture Collection) were grown, infected, and selected as described by Sirvent and colleagues (21). This procedure allows the generation of stable expressors in a polyclonal background. Src expressors were isolated by fluorescence-activated cell sorting and shRNA-expressing cells by puromycin (1  $\mu$ g/mL) selection. Standard cell growth was performed in 2% serum medium and measured by Sulforhodamide B staining (Sigma Aldrich). Soft-agar cell growth was performed as in Sirvent and colleagues (21). After 18 to 21 d, colonies having >50 cells were scored as positive. Cells were treated every day for standard conditions and every week for soft-agar assays, with vehicle or indicated drug. Cell migration and invasion assays were performed in Boyden chambers (BD Bioscience) using 50,000 and 100,000 cells, respectively, and in the presence of 100  $\mu$ L of Matrigel (2 mg/mL; BD Bioscience) for invasion assays.

**Growth of SW620 xenografts in nude mice.** *In vivo* experiments were performed in compliance with the French guidelines for experimental animal studies (Direction des Services Vétérinaires, Ministère de l'Agriculture, agreement no. B 34-172-27) and fulfill the U.K. Coordinating Committee on Cancer Research guidelines for the welfare of animals in experimental neoplasia. Swiss *nu/nu* (nude) mice (Charles River) were injected s.c. with  $2.10^6$  of SW620 cells infected with the control virus on one flank and with the same amount of cells infected with the Src virus on the opposite side. Tumor growth was assessed by tumor volume and tumor mass measurement at indicated days.

**MS analysis.** SILAC ( $^{13}\text{C}_6^{15}\text{N}_4$ -Arg- and  $^{13}\text{C}_6^{15}\text{N}_2$ -Lys as heavy labeling), phosphotyrosine immunoaffinity purification (using a mixture of a mixture of 4G10 and pY100 antibodies), and tryptic digests were essentially performed as described by Amanchy and colleagues (22), except that cells ( $2.10^8$ ) were treated with the tyrosine phosphatases inhibitor orthovanadate (1 mmol/L) for 1 h before lysis to increase tyrosine phosphorylation content.  $\beta$ -Octylglucoside (50 mmol/L) was also added in the 1% Triton X-100 lysis buffer to increase protein solubilization. Purified proteins were next separated on a SDS-PAGE gel. Trypsin-digested samples (1  $\mu$ L) obtained from 44 cut-gel slices were next analyzed online using nanoflow high performance liquid chromatography-nanoelectrospray ionization on a quadrupole time-of-flight mass spectrometer (QSTAR Pulsar-I; Applied Biosystems) coupled with an Ultimate 3000 high performance liquid chromatography (Dionex). Desalting and preconcentration of samples were effected on-line on a Pepmap precolumn (0.3  $\times$  10 mm). A gradient consisting of 0% to 40% B in 60 min, 80% B in 15 min (A, 0.1% formic acid, 2% acetonitrile in water; B, 0.1% formic acid in acetonitrile) at 300 nL/min was used to elute peptides from the capillary (0.075  $\times$  150 mm) reverse-phase column (Pepmap; Dionex), fitted with an uncoated silica PicoTip Emitter (NewObjective) with an outlet diameter of 8  $\mu$ m. Spectra were recorded using the Analyst QS 1.1 software (Applied Biosystems). Variables were adjusted as follows: ion spray voltage, 1800 V; curtain gas, 25; declustering potential, 75 V; focusing potential, 265 V; declustering potential 2, 15 V. Spectra were acquired with the instrument operating in the information-dependant acquisition mode throughout the high performance liquid chromatography gradient. Every 7 s, the instrument cycled through acquisition of a full-scan spectrum (1 s) and 2 MS/MS spectra (3 s each). Peptides fragmentation was performed using nitrogen gas on the most abundant doubly or triply charged ions detected in the initial MS scan, with a collision energy profile optimized according to peptide mass (using manufacturer variables), and an active exclusion time of 0.60 min. All MS/MS spectra were searched against the *Homo sapiens* entries of either



**Figure 2.** Src phosphoproteomic analysis in SW620 cells using SILAC. *A*, Western blot showing the tyrosine phosphorylation content of cells infected with the shown retroviruses and treated or not with orthovanadate as indicated. Western blotting from indicated p-Tyr immunoprecipitations. *B*, quantification of phosphorylations by SILAC. Summary of fold change with Src for 467 proteins shown in Supplementary Table S1, with several individual proteins indicated. Proteins with ratio  $>1.3$  (*top line*) are considered as increased and  $<0.7$  (*bottom line*) as decreased in their tyrosine phosphorylation content.

Swiss-Prot or Trembl databases (release 53.0)<sup>4</sup>, by using the Mascot v 2.1 algorithm.<sup>5</sup> Search variables were mass accuracy 0.1 Da for MS and MS/MS data; 1 miscleavage; variable modifications: oxidized methionine, Phospho (Y), SILAC-labels: Lys-8 and Arg-10. All significant hits ( $P < 0.05$ ) were manually inspected. Quantification was performed by using MSQuant v1.4.1 software developed by Mann and colleagues.<sup>6</sup> Data were manually inspected and corrected when necessary. All significant hits were manually inspected.

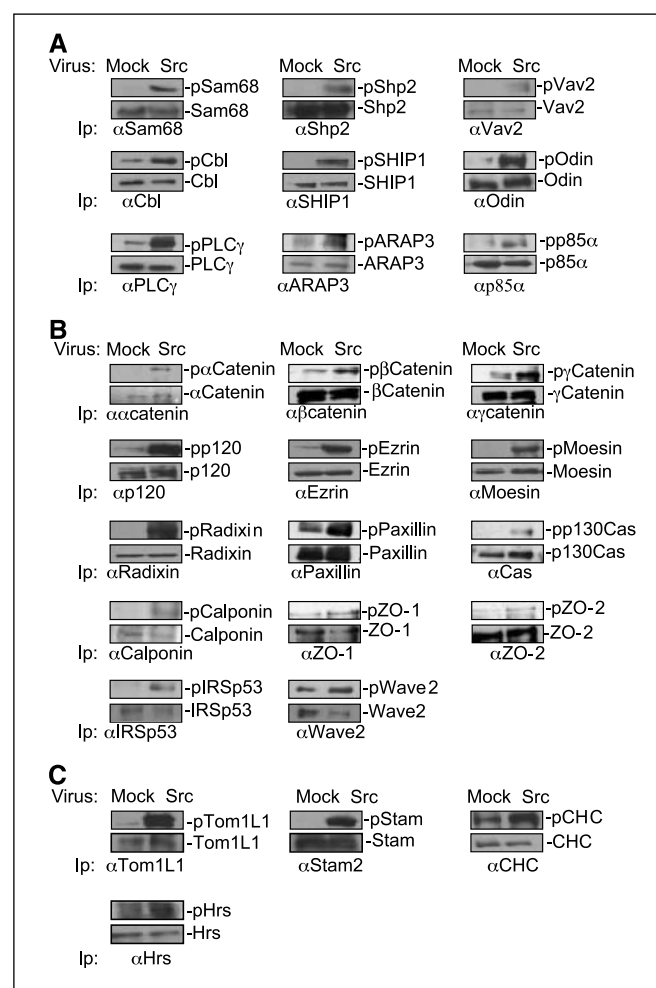
**Biochemistry.** Immunoprecipitation, Western blotting and siRNA transfections were performed as described in (21). Immunoprecipitates (*ip*, when indicated) or 20 to 50  $\mu$ g of proteins of a whole cell lysate were loaded per lane on 9% SDS-PAGE gels and then transferred onto Immobilon membranes (Millipore) as previously described. Detection was performed using the enhanced chemiluminescence system (Amersham). Optimal exposure times of membranes were used and protein expression and phosphorylation was quantified using ImageQuant TL software (Molecular Dynamics), and adjusted for background noise and protein loading.

## Results

**Src promotes growth and invasiveness of the SW620 CRC cells.** We first transduced wild-type Src retrovirally in the metastatic CRC SW620 cells that exhibit a moderate level of endogenous Src compared with the metastatic Colo205 cells (23). A  $>5$ -fold increase in Src protein level was obtained (Fig. 1A), which

induced a strong increase in tyrosine phosphorylation content (Fig. 2A) together with significant cellular oncogenic activity: increased cell growth was observed both in standard (Fig. 1B) and soft agar conditions (Fig. 1C). Similarly, Src increased tumor growth by 3-fold in mouse xenograft assays (Supplementary Fig. S1). Although Src moderately increased cell motility (Fig. 1D, *left*), it induced remarkable cell invasiveness (Fig. 1D, *right*). This cellular activity was still dependent on Src activity as it was inhibited by the Src-like inhibitor SU6656 (Fig. 1D, *right*; ref. 24). These data supports the hypothesis that the induction of the Src level is sufficient to induce its oncogenic activity even at late stage of tumorigenesis.

**Quantitative Src phosphoproteomics in CRC cells.** We next sought Src substrates important for oncogenic signaling by quantitative phosphoproteomics using the SILAC approach. SW620-Src cells were labeled with “heavy”  $^{13}\text{C}_6^{15}\text{N}_4$ -Arg- and  $^{13}\text{C}_6^{15}\text{N}_2$ -Lys-containing medium, whereas SW620 cells were cultured in “light” normal conditions. Cell lysates were next combined, and phosphotyrosine-containing proteins were purified using antiphosphotyrosine antibodies. Proteins were separated by

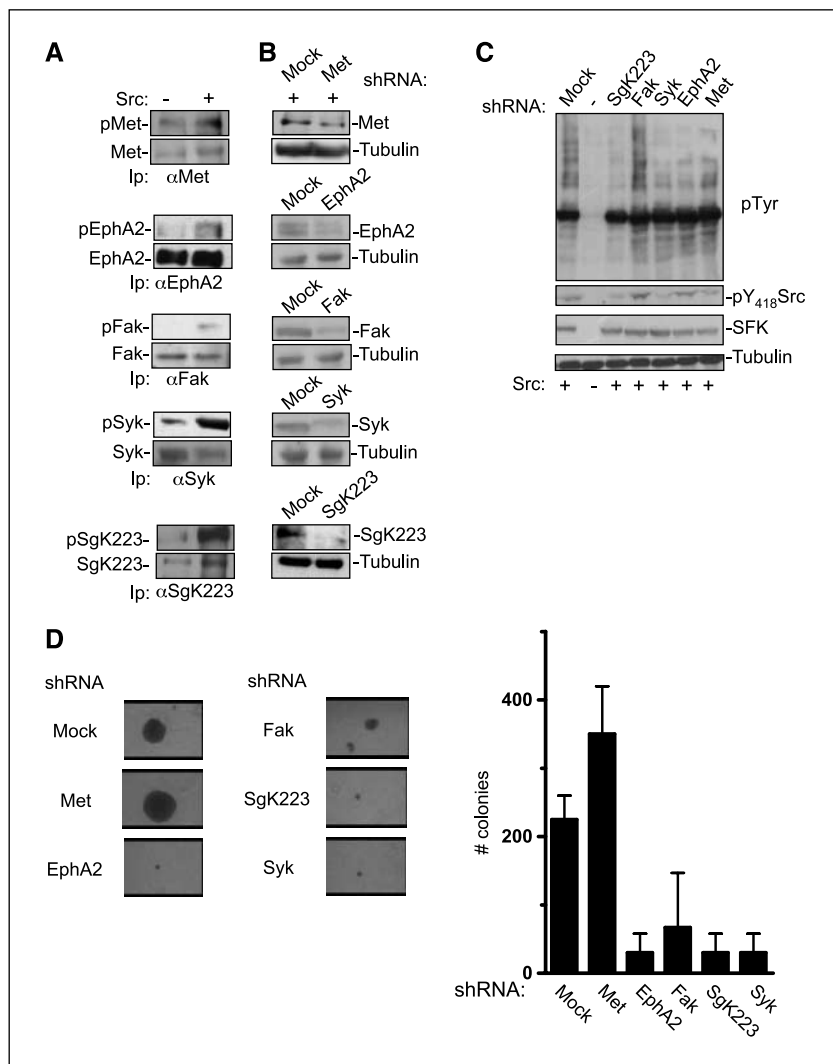


**Figure 3.** Validation of Src-induced protein tyrosine phosphorylation by immunoprecipitation and Western blotting. Level and tyrosine phosphorylation content of selected Src targets as identified by phosphoproteomics. Western blotting analysis of tyrosine phosphorylation content and level of indicated protein that was immunoprecipitated from vanadate-treated cells infected with shown virus and using indicated antibodies. *A*, signaling proteins; *B*, cytoskeletal-associated proteins; *C*, vesicular trafficking-associated proteins.

<sup>4</sup> <http://www.expasy.ch>

<sup>5</sup> <http://www.matrixscience.com>

<sup>6</sup> <http://msquant.sourceforge.net>



**Figure 4.** Src targets a cluster of tyrosine kinases important for increased tyrosine phosphorylation content and the promotion of anchorage-independent cell growth. *A*, tyrosine kinases with increased tyrosine phosphorylation content upon Src expression. Western blot showing the tyrosine phosphorylation and the protein levels in indicated tyrosine kinase immunoprecipitates from cells overexpressing Src or not, as shown. *B*, Western blot showing the level of indicated tyrosine kinases obtained from lysates of SW620-Src cells infected with retrovirus expressing the indicated shRNA. The level of tubulin is also shown. *C*, Western blot showing the tyrosine phosphorylation content of cells overexpressing or not Src as shown and infected with indicated shRNA. *D*, example (left) and statistical analysis (right) of anchorage-independent cell growth (number of colonies) of SW620-Src cells infected with retroviruses expressing the indicated shRNA. Columns, mean ( $n > 3$ ); bars, SD.

SDS-PAGE, digested with trypsin, and subjected to liquid chromatography-tandem MS (22). Quantification was made from the relative intensities of Arg/Lys labeled tryptic peptides. With this approach, we obtained 467 proteins, 136 of which exhibiting increased, and 13 reduced, tyrosine phosphorylation, upon Src expression (Supplementary Table S1; Fig. 2B). The small number of reduced phosphorylations was attributed to the cellular treatment of the tyrosine phosphatases inhibitor orthovanadate performed shortly before lysis to increase tyrosine phosphorylation content. Src-induced phosphorylation was next confirmed biochemically for 32 of 35 of them (Figs. 3 and 4A), suggesting that >90% of the identified candidates are indeed Src substrates and thus validates the SILAC analysis. The absence of increased tyrosine phosphorylation content for three of them may be due to an increased association with Src substrates or/and the inability of the used antibodies to recognize the phosphorylated form of the protein. Forty-one of identified proteins have signaling, and 31 cytoskeletal functions (Supplementary Table S2). Of some note are the CDCP1/PKC $\delta$  transmembrane signaling complex involved in survival (25), the E-cadherin/catenins complexes in mesenchyme induction and cell motility (2), and proteins of the ERM family in cell invasion (26). Additionally, Src targets the Wave2 and the  $\alpha 6/\beta 4$  integrin

complexes, recently identified as novel players of epithelial cell motility/invasion (27, 28). We also noticed a subgroup of signaling proteins that regulate the small GTPases of the Rho family, and which may modulate actinic cytoskeleton occurring during cell invasion (26). These include the Rho GAP ARAP1, 3 and p200 Rho GAP, and the Rho GEF Vav2 and FGD5, which has not been previously identified as a Src substrate. Unexpectedly, we found a third group of 23 Src targets with vesicular trafficking functions (Supplementary Table S2). This was exemplified with components of the COPII complex of the secretory pathway (29) and VHS-containing proteins (Hrs and Tom1L1), involved in transmembrane proteins trafficking/signaling (30).

**Src phosphorylates a cluster of tyrosine kinases important for cell invasiveness.** The SILAC analysis confirmed a 6-fold increase in Src expression showing accuracy of the method, but it additionally identified a cluster of 7 protein tyrosine kinases and pseudo-tyrosine kinases as potential Src substrates (Table 1). This included the receptors Met, EphA2, and EphB2, and the cytoplasmic tyrosine kinases Fak and Syk and pseudokinases SgK223 and SgK269. Increased tyrosine phosphorylation was confirmed for 5 of them biochemically, i.e., Met, EphA2, Fak, Syk and SgK223 (Fig. 4A), and this correlated with a Src complex



**Table 1.** List of tyrosine kinases targeted by Src

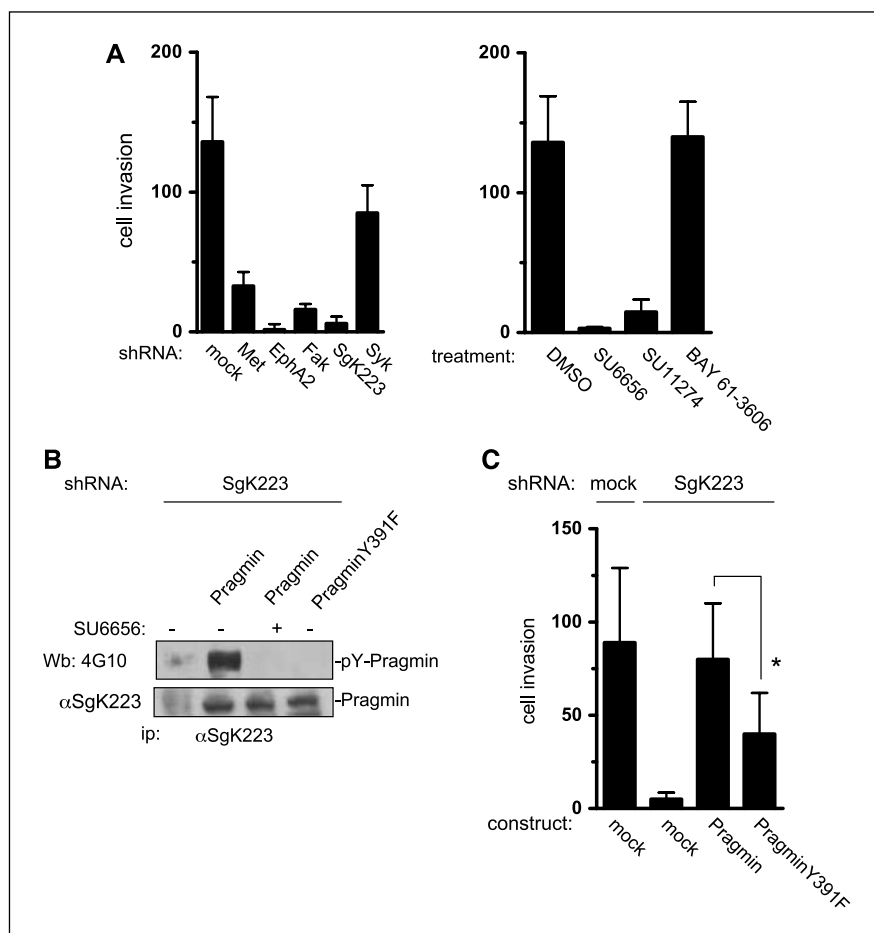
Tyrosine kinase	Fold change with Src expression
Src	6.27
Syk	4.44
EphA2	2.52
SgK223	2.34
Met	2.12
EphB2	1.74
SgK269	1.62

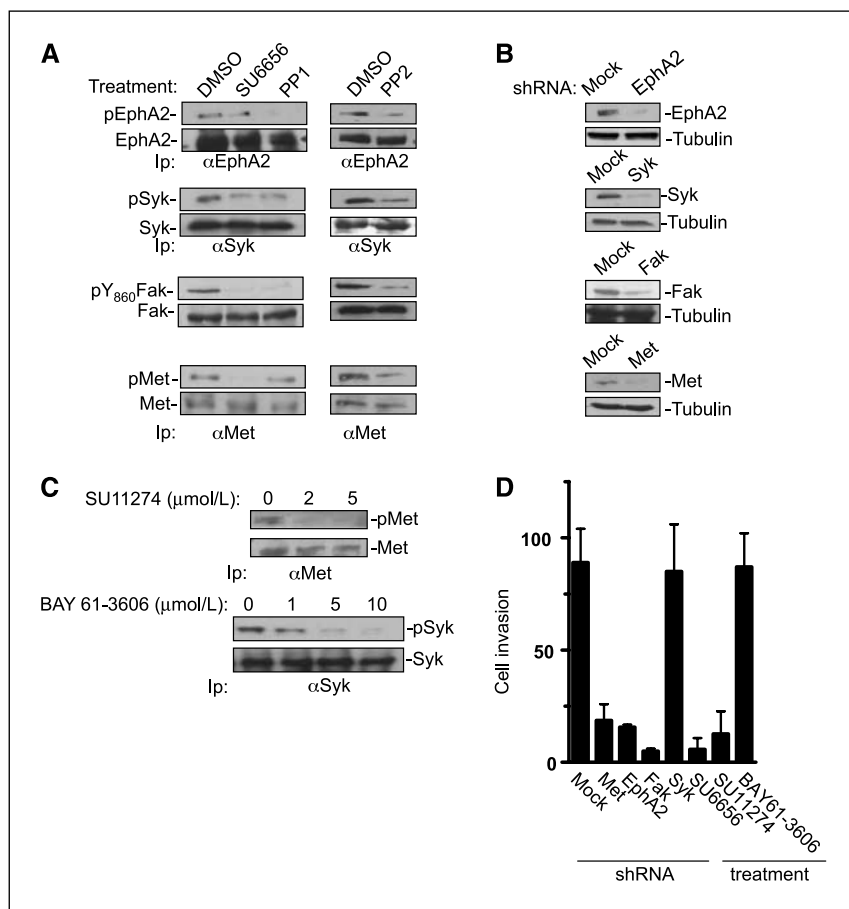
formation (data not shown). Similar results were obtained from cells that were not treated with the phosphatase inhibitor orthovanadate, indicating that these phosphorylations were specific to Src expression (Supplementary Fig. S2A). We next investigated their role in Src-induced tyrosine phosphorylation. To this end, respective tyrosine kinase was inactivated by stably expressing a shRNA leading to >50% reduction of both mRNA (data not shown) and protein levels (Fig. 4B) in SW620-Src cells. We observed that tyrosine phosphorylation was significantly reduced in cells with inactive SgK223 and Syk, and at a lower level in cells with inactive Met. Accordingly, Src-induced Met and Syk activation as shown by the increase level in the phosphorylated and activated form of these kinases (Supplementary Fig. S2B). Therefore Src may use these TyrK activities to induce maximal phosphorylation in

these CRC cells. Surprisingly, Src-specific activity was also reduced in these cells with inactive Met, EphA2, Syk, and SgK223, suggesting the existence of interplay between Src and these TyrK for signaling (Fig. 4C). The function for these kinases was next evaluated on Src oncogenic activity. We found that Src-induced anchorage-independent cell growth was strongly inhibited in all cell-lines, except for Met (Fig. 4D). However, none of them had a significant role in Src-induced cell proliferation (Supplementary Fig. S3), indicating that these TyrK may effect on survival signaling rather than cell-cycle progression, the later being probably ensured by phosphorylation of direct Src substrates.

The role for these TyrK was next evaluated on Src-induced invasiveness. We found that this cellular activity was strongly inhibited in all cell-lines with down-regulated tyrosine kinases, except Syk (Fig. 5A, left). Similar results were obtained from SW620-Src cells that were transiently transfected with specific siRNA targeting Met, EphA2, and Fak, indicating that these inhibitory effects were not due to off-targets and/or long-term depletion of these kinases (Supplementary Fig. S4C and D). In contrast, they had a low effect on the residual invasiveness of SW620 cells, confirming that their role is tightly related to Src activity (Supplementary Fig. S4A and B). The absence of Syk function was next confirmed with the orally available Syk catalytic inhibitor BAY 61-3606 (31), which did not affect invasiveness (Fig. 5A, right), while reducing Syk tyrosine phosphorylation (Fig. 6C, bottom). In contrast, the Met inhibitor SU11274 (Fig. 6C, top; ref. 32) blocked this cellular response (Fig. 5A, right), confirming the requirement of

**Figure 5.** Src targets a cluster of tyrosine kinases important to the promotion of cellular invasiveness. **A**, invasiveness of SW620-Src cells infected with retroviruses expressing the indicated shRNA (left). Right, invasiveness of SW620-Src cells treated with vehicle (DMSO), 5  $\mu$ mol/L SU6656, 2  $\mu$ mol/L SU11274, or 5  $\mu$ mol/L BAY 61-3606 as indicated. Number of cells per field that have invaded through Matrigel in Boyden chamber assays. **B**, Src phosphorylates the rat ortholog of SgK223, Pragmin, at Tyr391 in SW620-Src cells. Tyrosine phosphorylation and protein content of immunoprecipitated Pragmin mutants that were retrovirally transduced in SW620-Src cells expressing SgK223-specific shRNA as indicated. **C**, Pragmin but not PragminY391F restores invasiveness in SW620-Src cells with reduced SgK223. Number of cells per field that have invaded through Matrigel in Boyden chamber assays. Columns, mean ( $n > 3$ ); bars, SD.





**Figure 6.** Src targets a similar cluster of tyrosine kinases for the promotion of invasiveness of Colo205 cells. **A**, Src phosphorylates a similar cluster of tyrosine kinases in Colo205 cells. Western blot of the tyrosine phosphorylation content of shown immunoprecipitated tyrosine kinases from cells treated with indicated drugs (5  $\mu$ mol/L). **B**, Western blot showing the level of indicated tyrosine kinase obtained from lysates of Colo205 cells infected with retrovirus expressing the indicated shRNA. The level of tubulin is also shown. **C**, inhibition of Met and Syk phosphorylation by SU11274 and BAY 61-3606, respectively. Western blot showing the tyrosine phosphorylation and protein level of the indicated tyrosine kinases that were immunoprecipitated from Colo205 cells treated as indicated for 3 h. **D**, invasiveness of Colo205 cells infected with retroviruses expressing the indicated shRNA or treated with the indicated drugs (5  $\mu$ mol/L SU6656, 2  $\mu$ mol/L SU11274, or 5  $\mu$ mol/L BAY 61-3606). Number of cells per field that have invaded through Matrigel in Boyden chamber assays. Columns, mean ( $n > 3$ ); bars, SD.

Met in this Src oncogenic function. It should be mentioned that BAY 61-3606 strongly inhibited cellular proliferation, in contrast to what observed in cells with reduced Syk (Supplementary Fig. S3). This compound may therefore target an additional kinase important for cellular proliferation to be identified.

Because very few information is available on SgK223, we wished to address its invasive function in more details. To this end, the rat ortholog Pragmin was reintroduced in SW620-Src cells with reduced Sgk223, which was not targeted with the used shRNA sequence. Pragmin largely restored Src invasive activity, confirming a specific function for SgK223 in this signaling process (Fig. 5B and C). Whether this uncovered function was regulated by Src was next investigated. The phosphosite data base<sup>7</sup> revealed that SgK223 was frequently phosphorylated on Tyr413. Accordingly, we found that Src induced a robust phosphorylation of Pragmin in these cells, unlike PragminY391F, in which the corresponding Tyr413 present in Pragmin was replaced by Phe (Fig. 5B). At the functional level, PragminY391F had a reduced rescuing effect in these cells (Fig. 5C). This set of data strongly suggested that Src invasive signaling implicates phosphorylation of SgK223 on Tyr413 in advanced CRC cells.

Finally, we wished to confirm these data in cells expressing high endogenous Src, with the example of the metastatic CRC cell-line Colo205. As observed with SW620-Src, these cells required a Src-like activity to induce cellular invasion (Fig. 6D). We found that the tyrosine kinases Met, EphA2, Fak, and Syk were also phosphory-

lated in a Src-like dependent manner, as suggested with the Src-like inhibitors SU6656, PP1, and PP2 (Fig. 6A). The variable effect of SU6656 and PP1 on these biochemical events were attributed to their capacity to target distinct pools of Src present in these cells rather than the targeting of additional kinases (data not shown). SgK223 protein phosphorylation could not be assessed in these cells. This may probably due to the moderate efficacy of our antibodies and the low level of the expressed protein as observed on its mRNA level (data not shown). At the functional level, all these phosphorylated TyrK, but not Syk, were required for cell invasion/down-regulation of respective kinases (Fig. 6B) with the same shRNA strategy gave strong inhibitory effects (Fig. 6D). Thus, Src uses a similar TyrK network to induce its invasive activity in Colo205 cells, suggesting the existence of a common TyrK signaling network for oncogenic induction in advanced CRC cells.

## Discussion

Here, we provide new insights into Src oncogenic activity in CRC. First, our data show that normal Src overexpression can induce high invasiveness of advanced cancer cells *in vitro*, in close correlation with the proposed role for Src in CRC metastasis (3). Second, applied quantitative SILAC phosphoproteomics uncovered several features of Src signaling in these cells, including phosphorylation/interaction of components of phosphoinositides and Rho GTPases-dependent pathways and regulators of filamentous actin (F-actin), microtubule, and vesicular trafficking networks. These data also show that Src targets more than a dozen

<sup>7</sup> <http://www.phosphosite.org>

of plasma membrane proteins, several of them having ligand, receptor/coreceptor functions related to cellular growth, survival, and adhesion. Therefore, Src may use a wide range of reverse signaling processes important for its oncogenic activity as recently suggested with the Met receptor (5).

Another important insight from this study relies on the interaction of Src with a cluster of tyrosine kinases required for cell invasion. Src may thus require additional tyrosine kinases to phosphorylate components of the invasive pathway. This cluster comprises the receptors Met and EphA2, previously reported interacting with Src (33, 34) and being implicated in CRC tumorigenesis (35, 36). Moreover, our functional studies show that they mediate Src oncogenic activity in agreement with a reverse signaling process operating in these cells. It also includes the cytoplasmic tyrosine kinase Fak, which has been largely implicated in Src-induced cell motility and invasion (37). Interestingly, Fak does not effect on the global tyrosine phosphorylation content in contrast to the other members of the cluster, suggesting that either it phosphorylates a discrete group of substrates or it has an adaptor function, as previously reported (37). More surprisingly is the identification of Syk as a Src substrate in these cancer cells, which is inconsistent with its tumor suppressor role described in breast cancer (38). Therefore, Syk may have distinct functions dependent on the tumor origin. In supporting this idea, Syk regulates Src-induced tyrosine phosphorylation and anchorage-independent growth of SW620-Src cells. This is further supported with the function recently reported in squamous cell carcinoma of the head and neck (39). Finally, we show that Src also uses Sgk223 for its invasive activity. Sgk223 belongs to the 10% of catalytic-inactive kinases present in the human kinome (40) that nevertheless can have important oncogenic function. This has been first highlighted with the epidermal growth factor receptor (EGFR) member HER3 in breast cancer. HER3 exhibits a coreceptor function that boosts signaling induced by other receptors of the family (41). Very few information is however available for Sgk223. It belongs to the NFK3 subfamily that also includes Sgk269, with unknown functions (40). Interestingly, Sgk223 has been recently identified as an important effector of the small GTPase Rnd2 for Rho-dependent F-actin assembly (15), which would fit with its proinvasive activity in CRC cells. Although it does not have apparent intrinsic tyrosine kinase activity (data not shown), Sgk223 strongly effects on Src-induced tyrosine phosphorylation

in cellulo. We thus hypothesize that it regulates Src phosphorylation events important for Rho-dependent signaling. Overall, these results assign an unanticipated role for Sgk223 in Src oncogenic signaling and suggest an involvement of this pseudo-kinase in CRC advanced tumorigenesis.

Finally, our report suggests that Src orchestrates a tyrosine kinases signaling network for invasiveness. This notion has been recently suggested with the example of two recent phosphoproteomic studies revealing the phosphorylation of cluster of the TyrK FAK, EphA2, Met, and ACK in Src-transformed mouse fibroblasts (42) and the cluster of TyrK including Met and EphA2 in non-small cell lung cancer lines expressing activated ROS, ALK, PDGFR $\alpha$ , and EGFR (43). Nevertheless, the function for these TyrK networks has not been investigated. These reports together with our results thus suggest the involvement of a common TyrK signaling network operating during neoplastic transformation initiated by a deregulated TyrK. Interestingly, our data additionally indicate that Met, Syk, and Sgk223 may in turn further activate Src, uncovering an interplay between Src and these tyrosine kinases. How Src interacts with these tyrosine kinases is an important issue that can be addressed by phosphoproteomics. Similarly, these results raise the interesting idea that Src deregulation could originate, at least in part, from the complex array of phosphorylated/activated tyrosine kinase receptors recently observed in other solid tumors with important oncogenic activities (44); thus targeting Src activity could be of therapeutic value in these cancers.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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