Adaptor Protein Crk Induces Src-Dependent Activation of p38 MAPK in Regulation of Synovial Sarcoma Cell Proliferation

Takuya Watanabe,¹ Masumi Tsuda,² Shinya Tanaka,³ Yusuke Ohba,³ Hideaki Kawaguchi,³ Tokifumi Majima,² Hirofumi Sawa,⁵ and Akio Minami¹

Departments of ¹Orthopedic Surgery and ²Joint Replacement and Tissue Engineering and Laboratories of ³Pathophysiology and Signal Transduction and ⁴Cancer Research, Hokkaido University Graduate School of Medicine; ⁵Department of Molecular Pathobiology, Hokkaido University Research Center for Zoonosis Control, Sapporo, Japan

Abstract
The adaptor protein Crk mediates intracellular signaling related to cell motility and proliferation and is implicated in human tumorigenesis. The role of Crk in the growth of human sarcoma has remained unclear, however. The present study shows that Crk-induced activation of Src and subsequent signaling by p38 mitogen-activated protein kinase (MAPK) contribute to the enhanced proliferation of human synovial sarcoma cells. Depletion of Crk by RNA interference markedly inhibited proliferation of the synovial sarcoma cell lines HS-SYII, SYO-1, and Fuji as well as prevented anchorage-independent growth. Conversely, reconstitution with CrkII by authentic small interfering RNA–resistant Crk gene restored proliferation in Crk-silenced SYO-1 cells. Crk-depleted synovial sarcoma cells manifested enhanced transcriptional activity and expression of the p16INK4A gene, resulting in their accumulation in G1 phase of the cell cycle. In response to hepatocyte growth factor stimulation, Crk prominently induced the tyrosine phosphorylation of Grb2-associated binder 1 through activation of Src and focal adhesion kinase, and the Src family kinase inhibitor PP2 almost completely inhibited the proliferation of SYO-1 cells. Crk also induced the phosphorylation of p38 MAPK, and SB203580, a p38 MAPK–specific inhibitor, increased expression of p16INK4A gene in SYO-1 cells. Furthermore, SB203580 or depletion of p38 MAPK by small interfering RNA suppressed both the phosphorylation of Akt triggered by hepatocyte growth factor and the proliferation of SYO-1 cells. These results suggest that Crk promotes proliferation of human synovial sarcoma cells through activation of Src and its downstream signaling by a novel p38 MAPK-Akt pathway, with these signaling molecules providing potent new targets for molecular therapeutics. (Mol Cancer Res 2009;7(9):1582–92)

Introduction
The signaling adaptor protein Crk mediates diverse cellular responses including proliferation, differentiation, and migration. Crk was originally isolated as the product, v-Crk, of an avian sarcoma virus CT1 oncogene (1). Its mammalian homologue exists in two alternatively spliced isoforms: CrkII, which is composed of one SH2 domain and two SH3 domains [SH2-SH3(N)-SH3(C)], and CrkI, which contains one SH2 domain and one SH3 domain (SH2-SH3), similar to v-Crk (2). Crk transmits signals from various tyrosine-phosphorylated proteins, including components of focal adhesions, growth factor receptors, and signaling scaffold proteins, by binding to them via its SH2 domain (3). It subsequently associates with guanine nucleotide exchange factors, such as DOCK180 and C3G, via its SH3(N) domain (4, 5). DOCK180 and its binding molecule ELMO cooperatively regulate the activity of the small GTPase Rac1 and are thereby thought to control cell motility (3). C3G controls cell adhesion and cell proliferation by activating Rap1 and R-Ras, respectively (6). In addition to its physiologic functions, Crk contributes to the malignant conversion and progression of tumor cells. Overexpression of Crk has been detected in many types of human cancer cells, especially in those derived from tumors with poor prognosis such as brain, breast, and ovarian tumors as well as synovial sarcoma (7-10). The precise pathologic role of Crk in these cancer cells has remained unclear, however.

Synovial sarcoma is a high-grade malignant tumor of soft tissue with a poor prognosis and accounts for 7% to 10% of all malignant soft-tissue tumors (11). It arises primarily in the extremities of young adults (12), especially in the periarticular region, and it often metastasizes to the lung. Given that both the receptor tyrosine kinase c-Met and its ligand, hepatocyte growth factor (HGF), are highly expressed in synovial sarcoma (13), the autocrine activation of signaling by this receptor-ligand pair is implicated in tumorigenesis and progression of this sarcoma.

We have shown previously that, in response to HGF stimulation, Crk-mediated sustained phosphorylation of the c-Met docking protein Grb2-associated binder 1 (Gab1) evokes prominent activation of Rac1 and thereby promotes the migration of human synovial sarcoma cells (7). Overexpression of Crk in

Received 2/18/09; revised 7/7/09; accepted 7/14/09; published OnlineFirst 9/8/09.


The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Masumi Tsuda, Laboratory of Pathophysiology and Signal Transduction, Hokkaido University Graduate School of Medicine, N15, W7, Kitaku, Sapporo 060-8638, Japan. Phone: 81-11-706-5158; Fax: 81-11-706-7877. E-mail: tsudam@med.hokudai.ac.jp

Copyright © 2009 American Association for Cancer Research.
doi:10.1158/1541-7786.MCR-09-0064

Mol Cancer Res 2009;7(9). September 2009
293T cells also induced tyrosine phosphorylation of Gab1 in the absence of extracellular stimuli (14). Given that Crk is abundant in synovial sarcoma cells (7), Crk-mediated persistent phosphorylation of Gab1 might be expected to contribute to the pathology of this tumor. We have therefore now examined the possible role of Crk in the proliferation of human synovial sarcoma cells. We found that the Crk-induced phosphorylation of Gab1 is mediated by Src and focal adhesion kinase and results in the activation of a novel signaling pathway mediated by the mitogen-activated protein kinase (MAPK) p38 and subsequent Akt. Depletion of Crk or inhibition of Src family kinases or of p38 MAPK resulted in marked suppression of the proliferation of synovial sarcoma cells. Crk thus appears to contribute to the malignant potential of human synovial sarcoma cells by regulating not only cell motility but also cell proliferation.

Results
Depletion of Crk Inhibits the Proliferation and Anchorage-Independent Growth of Synovial Sarcoma Cells

Crk is abundant in human synovial sarcoma cells (7). We established human synovial sarcoma cell lines depleted of Crk by RNA interference (RNAi) to evaluate the functions of this protein. RNAi resulted in depletion of both CrkI and CrkII, but not that of CrkL, in the independent human synovial sarcoma cell lines HS-SYII, SYO-1, and Fuji (Fig. 1A; Supplementary Fig. S1A). The proliferation of Crk-silenced HS-SYII cells, in which CrkI and CrkII were almost completely eliminated (Fig. 1A), was found to be markedly inhibited compared with that of the parental cells or of HS-SYII cells transfected with a vector encoding a control small interfering RNA (siRNA; Fig. 1B). Of three clones of Crk-silenced SYO-1 cells, CrkI-2 manifested a substantial reduction in proliferation (Fig. 1B) and the complete depletion of anchorage-independent growth in a colony formation assay in soft agar (Fig. 1C; Supplementary Fig. S2), whereas the proliferation of CrkI-1 or CrkI-3 was similar to that of the parental cells (Fig. 1B). Given that the two latter clones still expressed a small amount of CrkII (Fig. 1A), these results suggested that CrkII plays an important role in the proliferation of SYO-1 cells. Consistently, reconstitution of CrkII by authentic siRNA-resistant Crk gene resulted in an almost complete restoration in the proliferation of CrkI-2 cells (Fig. 1D). On the other hand, CrkI is also implicated in the proliferation of synovial sarcoma cells in a cell context–dependent manner. Proliferation and anchorage-independent growth of the one clone (Crki) of Crk-depleted Fuji cells obtained was remarkably inhibited relative to that of the parental cells (Supplementary Fig. S1B and C). Three clones of Fuji cells transfected with a vector for a control siRNA (clones C-1 to C-3) unpredictably manifested a reduction in the abundance of CrkI but not in that of CrkII (Supplementary Fig. S1A); the proliferation in these cells was partially inhibited compared with those in parental cells (Supplementary Fig. S1B), which seems to be consistent with a previous report indicating a role of CrkI in transforming activity in 3Y1 rodent fibroblast (15). Supporting this finding, reconstitution of CrkII in Crk-silenced Fuji cells (Crki) resulted in the partial improvement in proliferation in spite of the sufficient expression of CrkII protein (Supplementary Fig. S1D).

Crk Down-Regulates p16INK4A Gene Expression through p38 MAPK in Synovial Sarcoma Cells

To identify targets of Crk-evoked signaling in regulation of synovial sarcoma cell proliferation, we next determined the expression levels of several genes related to cell cycle progression. Of the MAPKs extracellular signal-regulated kinase 1/2 (ERK1/2), p38 MAPK, and c-Jun NH2-terminal kinase (JNK), all of which are implicated in the growth of a range of malignancies, p38 MAPK specifically participates in synovial sarcoma cell proliferation (see below; Fig. 4C and D) as well as their spheroid formation (16); we thereby focused on p38 MAPK–mediated cell cycle regulators identified: cyclin D1, the phosphatase Cdc25A, and the cyclin-dependent kinase inhibitor p16INK4A (17–19). The amounts of cyclin D1 and Cdc25A mRNAs did not differ substantially between parental and Crk-silenced cells as measured by the conventional and quantitative real-time reverse transcription-PCR (RT-PCR) techniques (Fig. 2A). In contrast, the abundance of the mRNA for p16INK4A, which induces G1 arrest, was markedly increased by depletion of Crk (Fig. 2A). The MEK1/2 inhibitor U0126 and the p38 MAPK inhibitor SB203580 also increased the amount of p16INK4A mRNA in SYO-1 cells, with the effect of SB203580 being more pronounced than that of U0126 (Fig. 2B). A luciferase reporter assay revealed that the activity of the p16INK4A gene promoter was greater in Crk-silenced SYO-1 or Fuji cells than in the corresponding parental cells (Fig. 2C). Consistent with these findings, Crk depletion by RNAi resulted in an increase in the percentage of SYO-1 cells in G1 phase of the cell cycle and corresponding decreases in the numbers of cells in S and G2-M phases (Fig. 2D).

Furthermore, we examined the relation of Crk depletion and consequent up-regulation of p16INK4A gene expression to the development of synovial sarcoma in vivo. We showed previously that the ability of Fuji cells to form tumors in nude mice was impaired by Crk depletion (7). The amount of p16INK4A mRNA in tumors formed by Crk-silenced Fuji cells was greatly increased compared with that in those formed by parental cells (Fig. 2E), suggesting that Crk promotes the development of synovial sarcoma in vivo by regulating cell cycle progression.

Src Family Kinases Mediate the Crk-Induced Tyrosine Phosphorylation of Gab1 and the Cell Proliferation in Synovial Sarcoma Cells

Aberrant activation of HGF/c-Met signaling, due to high expressions in synovial sarcoma cells, has been implicated in key determinant of the oncogenic phenotypes of the tumor such as uncontrolled cell proliferation and invasion (13). Indeed, c-Met selective inhibitor SU11274 exhibited a reduction in the proliferation of SYO-1, HS-SYII, and Fuji cells in a dose-dependent manner (Supplementary Fig. S3), of which inhibitory effect is likely paralleled to the degree of c-Met activation in addition to the amount of this protein (7), supporting an implication of c-Met-derived signaling in the growth of synovial sarcoma. We thus examined the possible role of Crk in HGF/c-Met signaling of this tumor. We showed previously that Crk mediates sustained phosphorylation of Gab1 induced by HGF in human synovial sarcoma cells (7). Consistent with this finding, HGF-induced phosphorylation of Gab1 was partially inhibited in SYO-1 or Fuji cells depleted of Crk compared with that in the...
corresponding parental or control transfected cells (Fig. 3A; Supplementary Fig. S4). The expression of c-Met in SYO-1 cells was not affected by Crk depletion (Fig. 3A). In clarification of the mechanism of Gab1 phosphorylation induced by Crk in synovial sarcoma cells, we found that overexpression of wild-type (WT) CrkII in SYO-1 cells induced tyrosine phosphorylation of exogenous Gab1 even in the absence of external signaling (Fig. 3B), similar to our previous findings with 293T cells (14). Whereas expression of W169L or Y221F mutants of CrkII mimicked this effect of WT CrkII, that of the R38V mutant did not (Fig. 3B), indicating that the SH2 domain of CrkII is for Gab1 phosphorylation.

Our recent findings with 293T cells indicate that Crk has an ability to promote the kinase activity of Src through the possible association with Csk (14), leading to the induction of tyrosine phosphorylation of Gab1. Further supporting this finding, expression of a constitutively active mutant (Y527F) of Src in 293T cells, but not that of the WT protein, induced the phosphorylation of Gab1 in the absence of exogenous CrkII (Supplementary Fig. S5). Furthermore, although the WT protein clearly enhanced the phosphorylation of Gab1 in the presence of exogenous CrkII, this effect was more pronounced with the Y527F mutant. Conversely, dominant-negative mutants of Src (K295M) or focal adhesion kinase

**FIGURE 1.** Depletion of Crk results in inhibition of the proliferation of human synovial sarcoma cells. **A.** Establishment of Crk-silenced synovial sarcoma cell lines. HS-SYII and SYO-1 cells were transfected with vectors for a Crk-specific siRNA or a control siRNA and were subjected to selection to obtain permanent cell lines (designated Crki and C, respectively). Lysates of the transfected or parental (P) cells were subjected to immunoblot analysis with antibodies to Crk (for Crk and CrkII), CrkL, or pan-actin (loading control). **B.** Effect of Crk depletion on the proliferation of synovial sarcoma cells. Cells (1 x 10^5) were plated in culture dishes and counted at the indicated times thereafter. **C.** Effect of Crk depletion on anchorage-independent growth of SYO-1 cells. Parental, control transfected, or Crk-silenced (clone Crki-2) SYO-1 cells (2 x 10^5) were plated in 0.4% soft agar and incubated for 13 d, after which the number of colonies in the indicated size ranges was determined by microscopy. Top, representative micrographs; bottom, quantitative data. **D.** Effect of Crk complementation on the proliferation of Crk-silenced SYO-1 cells. Crk-silenced SYO-1 cells (Crki-2) were transfected with a vector encoding siRNA-resistant Rattus c-CrkII gene with Flag epitope tag or a control vector and were subjected to selection to obtain permanent cell lines (designated as Crki-R and Crki-C, respectively). Lysates of the transfected, parental, or Crk-silenced (Crki-2) cells were subjected to immunoblot analysis with antibodies to Crk or pan-actin (loading control; top). Cells (1 x 10^5) were plated in culture dishes and counted at the indicated times thereafter (bottom).
[hemagglutinin (HA) FRNK] moderately inhibited Gab1 phosphorylation induced by exogenous CrkII (Supplementary Fig. S5). These results thus suggested that the tyrosine kinases Src and focal adhesion kinase might mediate the phosphorylation of Gab1 induced by Crk in synovial sarcoma cells.

To examine the role of Src family kinases in the proliferation of synovial sarcoma cells, we determined the effect of an

---

**FIGURE 2.** Crk regulates cell cycle progression by inhibiting p16 INK4A gene expression through activation of p38 MAPK in synovial sarcoma cells. **A.** Crk-silenced SYO-1 (Crki-2), Fuji (Crki), and HS-SYII (Crki-2) cells as well as the corresponding parental cells were assayed for the amounts of cyclin D1, Cdc25A, p16INK4A, and GAPDH (internal control) mRNAs by conventional RT-PCR (left) and quantitative real-time RT-PCR analysis (right). In quantitative RT-PCR, data shown are mean of triplicate samples from three independent experiments. S, SYO-1 cells; F, Fuji cells; H, HS-SYII cells. *, P = 0.00222; **, P = 0.000995. **B.** SYO-1 cells were incubated for 3 or 6 h in the absence or presence of U0126 (10 μmol/L) or SB203580 (10 μmol/L) before determination of the abundance of p16INK4A and GAPDH mRNAs by RT-PCR analysis; the amount of p16INK4A mRNA was normalized by that of GAPDH mRNA and expressed relative to the normalized value for untreated cells. **C.** Activity of the p16 INK4A gene promoter in Crk-silenced SYO-1 (Crki-2) or Fuji (Crki) cells as well as in the corresponding parental cells was determined with a luciferase reporter assay; data represent normalized firefly luciferase activity expressed relative to that of parental cells and a mean±SD of triplicates from a representative experiment. Asterisks indicate P < 0.001. **D.** Cell cycle distribution of parental and Crk-silenced SYO-1 cells was also determined by flow cytometric analysis; the percentage of cells in G1, S, and G2/M phases of the cell cycle is indicated. **E.** Parental or Crk-silenced Fuji cells (Crki) were injected into the back of nude mice. Total RNA was isolated from the tumors that developed after 1 mo and was subjected to conventional RT-PCR analysis (left) and quantitative real-time RT-PCR analysis (right) of cyclin D1, Cdc25A, p16INK4A, and GAPDH mRNAs. Mean of two independent experiments. *, P = 0.0492.
inhibitor (PP2) of these kinases on the proliferation of SYO-1 cells. SYO-1 cell proliferation was almost completely inhibited in the presence of PP2 compared with that apparent in the presence of its inactive analogue PP3 (Fig. 3C). In addition, PP2 inhibited the residual proliferative activity of Crk-silenced SYO-1 cells (Fig. 3C). PP2 also inhibited Gab1 phosphorylation induced by HGF in SYO-1 cells (data not shown). Together, these results suggested that Crk-mediated activation of Src family kinases and subsequent Gab1 phosphorylation may contribute to the proliferation of synovial sarcoma cells.

**Association of Crk with DOCK180 Results in p38 MAPK Activation**

To explore the mechanism by which the Crk-Gab1 axis regulates synovial sarcoma cell proliferation, we next examined the phosphorylation status of the MAPKs ERK1/2, p38 MAPK, and JNK. HGF induced the phosphorylation of all three MAPKs in SYO-1 cells (Fig. 4A). Furthermore, the HGF-evoked phosphorylation of p38 MAPK, but not that of ERK1/2 or JNK, was specifically blocked in cells expressing the CrkII-R38V and CrkII-W169L mutants (Fig. 4A). Of note in which cells, the phosphorylation of p38 MAPK also decreased even at the quiescent state compared with those in the parental or WT CrkII-transfected SYO-1 cells (Fig. 4A), together indicating an implication of CrkII in p38 MAPK activation irrespective of HGF stimulation. To identify the mediators of Crk-dependent p38 MAPK phosphorylation, we further examined the phosphorylation of this kinase in 293T cells overexpressing DOCK180 or C3G. Phosphorylation of p38 MAPK was increased in cells overexpressing CrkII or DOCK180 but not in those overexpressing C3G, and coexpression of DOCK180, but not that of C3G, with CrkII and Gab1 further increased p38 MAPK phosphorylation (Supplementary Fig. S6A and B).

As expected, CrkII was found to bind to Gab1 and to DOCK180 through its SH2 and SH3(N) domains, respectively (Supplementary Fig. S7). The association of CrkII-R38V with DOCK180 was more pronounced than that of the WT protein (Supplementary Fig. S7) due to probably not to bind to the phosphotyrosine of CrkII itself. The association of CrkII-WT with C3G was undetectable in the presence of Gab1 (Supplementary Fig. S7), indicating that Crk may transmit signals from phosphorylated Gab1 preferentially to DOCK180. Consistent with these results, the levels of basal and HGF-induced phosphorylation of p38 MAPK in Crk-silenced SYO-1 cells (clone Crki-2) were reduced compared with those in the parental or control transfected cells (Fig. 4B). Depletion of Crk in SYO-1 cells also resulted in a slight promotion and reduction in the levels of basal and HGF-induced ERK1/2 phosphorylation, respectively, but did not affect JNK phosphorylation (data not shown).

We next examined the possible role of p38 MAPK activation in the proliferation of synovial sarcoma cells. We found that the proliferation of SYO-1 cells was reduced by SB203580, a specific inhibitor of p38 MAPK (Fig. 4C). Furthermore, SB203580 eliminated the residual proliferative activity of Crk-silenced SYO-1 cells (Fig. 4C). We also found that the proliferation of SYO-1 cells was suppressed by depletion of p38 MAPK with its specific siRNA, which targets the human p38α MAPK mRNA, to a similar extent by SB203580 (Fig. 4D; Supplementary Fig. S8). These results suggested that Crk may contribute to the proliferation of synovial sarcoma cells by associating with DOCK180 and eliciting the activation of p38 MAPK.

**p38 MAPK Mediates Phosphorylation of Akt in Synovial Sarcoma Cells**

During the course of experiments to explore potential cross-talk between p38 MAPK and other signaling pathways,
we found that SB203580 inhibited the HGF-elicited phosphorylation of Akt on both Ser\(^{473}\) and Thr\(^{308}\) residues in SYO-1 cells (Fig. 5A). The phosphorylation of Akt in response to HGF was also suppressed to a similar extent by the Src family kinase inhibitor PP2, by the JNK inhibitor SP600125, and by the phosphoinositide 3-kinase inhibitor LY294002, but it was unaffected by U0126, an inhibitor of MEK1/2 that blocks signaling by its downstream target.
ERK1/2 (Fig. 5B). We observed similar effects of these various inhibitors on HGF-induced Akt phosphorylation in Fuji cells (Supplementary Fig. S9). The HGF-induced phosphorylation of Akt was also inhibited clearly in Crk-silenced SYO-1 cells (Fig. 5C) and partially in p38α MAPK-eliminated SYO-1 cells (Fig. 5D). These results suggested that Akt signaling operates, at least partly, downstream of p38 MAPK in synovial sarcoma cells.

**FIGURE 5.** Phosphorylation of Akt mediated by p38 MAPK in synovial sarcoma cells. A. SYO-1 cells were incubated for 2 h in the absence or presence of SB203580 (10 μmol/L) and then for 30 min in the additional absence or presence of HGF (50 ng/mL). Cell lysates were then subjected to immunoblot analysis with antibodies to phosphorylated forms of Gab1, ERK1/2, or Akt (Thr308 or Ser473). The intensity of the band corresponding to the Ser473-phosphorylated form of Akt was quantified by image analysis and expressed relative to that for cells not exposed to SB203580 or HGF. B. SYO-1 cells were incubated for 2 h in the absence or presence of the indicated inhibitors (each at 10 μmol/L) and then for 30 min in the additional absence or presence of HGF (50 ng/mL). Cell lysates were then subjected to immunoblot analysis with antibodies to the indicated proteins (top). The ratio of Ser473-phosphorylated/total Akt band intensity was determined by image analysis and expressed relative to that for cells not exposed to inhibitor or HGF (bottom). C. Parental, control transfected, and Crk-silenced (Crki-2) SYO-1 cells were incubated in the absence or presence of HGF (50 ng/mL) for 30 min, lysed, and subjected to immunoblot analysis. D. SYO-1 cells were transfected with or without a p38α MAPK-targeting siRNA or a control siRNA. After 3 d of transfection, cells were incubated in the absence or presence of HGF (50 ng/mL) for 30 min, lysed, and subjected to immunoblot analysis with antibodies to phospho-Akt (Ser473), total Akt, or p38 MAPK. The intensity of the band corresponding to the Ser473-phosphorylated form of Akt was quantified by image analysis and expressed relative to that for cells without siRNA transfection and with HGF.
Discussion

Synovial sarcoma is a high-grade malignant tumor of soft tissue (11). Surgical resection with adjuvant radiotherapy or chemotherapy is the principal mode of treatment for synovial sarcoma, but the prognosis of affected individuals remains relatively poor because of a low response rate to conventional chemotherapeutic agents. The survival rate of patients with synovial sarcoma is only 35% to 50% at 5 years and 10% to 30% at 10 years. The development of therapeutics with a higher efficacy is thus urgently needed. Molecular therapeutics targeting protein tyrosine kinases is an attractive strategy, and expression profiling studies have identified several protein tyrosine kinases that are highly expressed in synovial sarcoma, including the epidermal growth factor receptor, fibroblast growth factor receptor 3, c-Kit, and human epidermal growth factor 2 (20-23); none or their specific inhibitors however, were found to substantially inhibit the proliferation of SYO-1 or Fuji cells (24), which suggests the peculiar characteristics of this tumor.

In the present study, we hereby unveil the role of Crk and its derivative novel signaling, Crk-Src-p38-p16INK4A, in oncogenesis of synovial sarcoma cells, which are available in both the presence and the absence of external HGF stimulation (Supplementary Fig. S10A and B). All of the depletion of Crk by RNAi, inhibition of Src family kinases (PP2), and p38 MAPK (SB203580) indeed suppressed the proliferation of synovial sarcoma cells even in the absence of HGF stimulation (Supplementary Fig. S10A). Although the HGF receptor c-Met, to some extent, contributes to the growth of synovial sarcoma cells, the Crk-mediated pathway seems to be independent of c-Met signaling under cell culture condition—absence of HGF—where SU11274, c-Met specific inhibitor, had no effect on p16INK4A induction (data not shown). On HGF stimulation, Crk-mediated signaling is engaged in the c-Met pathway, which is resulted form Gab1 recruitment by c-Met (Supplementary Fig. S10B). In addition, Crk-mediated Src activation, through the positive feedback mechanism, should be more elevated than that without HGF because of the synergistic activation of Src evoked by the c-Met-HGF pair, which leads to the persistent phosphorylation of Gab1 and consequent signaling even after the degradation of c-Met, consistent with our previous study (7). In fact, a single inhibition of c-Met per se was insufficient to reduce the viability of HS-SYII or Fuji cells (7, 25). Given that Crk is abundant in human synovial sarcoma cells (7), Crk-dependent persistent phosphorylation of Gab1 mediated through Src activation may underlie the malignant potential of this tumor type, with Src being a promising potential target for molecular therapeutics.

We have now shown that a novel DOCK180-p38 MAPK signaling pathway operates downstream of Crk in the regulation of synovial sarcoma cell proliferation. Crk plays important and distinct roles in the pathogenesis of diverse human cancers. CrkI is thought to function as a constitutively active form, whereas CrkII is under the control of tyrosine kinases as a result of intracellular binding of its SH2 domain to its phosphorylated Tyr221 residue (26). CrkI, but not CrkII, exhibits transforming activity in 3Y1 rodent fibroblasts (15). In synovial sarcoma, however, our results suggest that CrkII also promotes cell proliferation in a context-dependent manner: whereas CrkII was required for the proliferation of SYO-1 cells, CrkI appeared to play the dominant role in promoting the proliferation of Fuji cells. Our results thus implicate both CrkI and CrkII in regulation of the proliferation of synovial sarcoma cells.

Crk-induced Rac activation through DOCK180 has been well established in the regulation of cell movement (27), whereas a C3G-R-Ras-JNK signaling pathway is thought to operate downstream of Crk in control of cell cycle progression (28). However, a role for Crk in p38 MAPK signaling has not yet been shown. We showed previously that Crk plays an important role in HGF-induced Rac1 activation and the consequent enhancement of cell motility in synovial sarcoma cells (7). In the present study, we found that DOCK180, but not C3G, mediates Crk-dependent p38 MAPK phosphorylation and subsequent enhancement of cell proliferation. Our preliminary experiments indicate that this pathway is transmitted by Rac1 (data not shown). Rac is thought to contribute to the regulation of gene transcription and cell cycle progress through p38 MAPK and JNK signaling pathways in addition to its canonical function of the formation of lamellipodia and membrane ruffles (29, 30). Together, our observations suggest that Crk-DOCK180-Rac1 signaling may play a pivotal role in the regulation of both motility and proliferation in synovial sarcoma cells. Rac1 thus acts as a molecular switch to control distinct biological events, such as reorganization of the actin cytoskeleton and gene transcription, through different downstream effectors. In synovial sarcoma cells, signal transmission from Rac1 to p38 MAPK is likely mediated by PAK and MKK3/6 (31, 32).

Signaling by p38 MAPK is activated in response to cellular stress to block cell proliferation, to promote apoptosis, or to induce premature senescence (33). Furthermore, activation of p38 MAPK negatively regulates malignant transformation by down-regulating cyclin D expression (17), inhibiting the activity of Cdc25 (18), and up-regulating the expression of cyclin-dependent kinase inhibitors such as p16INK4A and p21WAF1 (19, 34). However, the effects of HGF-induced p38 MAPK activation on cell proliferation appear to be cell type specific. For instance, HGF promotes the proliferation of melanoma cells via p38 MAPK, ATF2, and cyclin D1 (35). In addition, p38 MAPK activation is essential for HGF-induced mitogenesis in lung adenocarcinoma cells (36). Our results now indicate that HGF-induced p38 MAPK activation results in down-regulation of p16INK4A gene expression and thereby promotes cell proliferation in synovial sarcoma cells. We showed previously that, under certain conditions, synovial sarcoma cells undergo premature senescence as a result of up-regulation of p21WAF1 gene expression mediated by the transcription factor Sp1 (37). Together, these various observations suggest that synovial sarcoma is a peculiar malignancy steering cells toward mitosis, senescence, or apoptosis in response to cell exposure to various extracellular stimuli.

Whereas our results suggest that Crk is indispensable for p38 MAPK activation induced by HGF in synovial sarcoma cells, both ERK1/2 and JNK were activated independently of Crk. Given that p38 MAPK activation also promotes HGF secretion (38), the resultant constitutive activation of signaling by this kinase may underlie the malignant potential of synovial sarcoma. Phosphorylation of p38 MAPK was found to be retained in spheroid cultures of SW982 synovial sarcoma cells (16), possibly consistent with our present results.
The expression of p16\textsuperscript{INK4A} is regulated at the transcriptional or post-transcriptional levels through deletion of the gene (39), methylation of the gene promoter (40), as well as changes in cyclin-dependent kinase 4/6 activity, Rd phosphorylation status (41), JunB expression level (42), and ERK pathway activity (43). Activated p38 MAPK is generally thought to up-regulate p16\textsuperscript{INK4A} expression (19), but we have now shown that it has the opposite effect in synovial sarcoma cells, an action that may be related to Akt signaling. In human ovarian cancer cells, phosphoinositide 3-kinase inhibits p16\textsuperscript{INK4A} expression through activation of an Akt-mammalian target of rapamycin (Tyr307) were from Cell Signaling; those to Gab1 were from Upstate; those to Flag (M2) were from Sigma; those to Myc (12CA5) to the HA epitope were from Roche; and those to pan-actin were from Chemicon. The Src family kinase inhibitor PP2 and its inactive analogue PP3, the JNK inhibitor SP600125, the phosphoinositide 3-kinase inhibitor LY294002, and the c-Met inhibitor SU11274 were obtained from Calbiochem; the p38 MAPK inhibitor SB203580 was from Invitrogen-BioSource; and the MEK1/2 inhibitor U0126 was from Cell Signaling. Human recombinant HGF was obtained from PeproTech.

**Immunoblot Analysis and Immunoprecipitation**

Immunoblot analysis and immunoprecipitation were done as described previously (7).

**Analysis of Cell Proliferation and Colony Formation Assay**

Analyses of cell proliferation and anchorage-independent growth by a colony formation assay were done as described (8). To assess the effects of Src family kinase or p38 MAPK inhibitors on cell proliferation, we exposed cells to 10 μmol/L PP2 or 10 μmol/L SB203580, respectively, after they had attached to the dish.

**Establishment of CrkII-Complemented Synovial Sarcoma Cell Lines**

Crk-silenced synovial sarcoma cells by RNAi (Crki-2 of SYO-1 and Crki of Fuji) were stably transected with a vector encoding siRNA-resistant \textit{Rattus} c-CrkII gene or the corresponding empty vector using Fugene HD reagents. Following selection with G418 at gradient step of 1 to 0.2 mg/mL (Sigma), expression levels of CrkII were confirmed by immunoblotting.

**Transfection of p38 MAPK-Specific siRNA**

SYO-1 cells were transfected with siRNA targeting the human p38α MAPK mRNA, but not p38β, γ, and δ (si-p38 MAPK; Qiagen), using HiPerFect reagent (Qiagen) according to the manufacturer’s instructions. AllStars Negative Control siRNA (si-Control; Qiagen) was used as a control.

**RNA Isolation and RT-PCR Analysis**

Total RNA was isolated from cells with the use of a RNeasy Mini Kit (Qiagen) and subjected to reverse transcription by SuperScript III reverse transcriptase (Invitrogen). The resulting cDNA was then subjected to the conventional PCR and quantitative real-time PCR with primers (forward and reverse, respectively) specific for human cyclin D1 (5-GCTGTGCATCTACACCGCAG-3 and 5-CTGAGGCTTTGCACGGAG-3), human Cdc25A (5-TGGACTCCAGGAGGGTAAAG-3 and 5-TTGAGCTTGTTCACCAGGAG-3), and human Cdc25B (5-TGGACATCCGAGGAAGTAA-3 and 5-TCTCTTCTATTTGGAAGGAACAT-3), human p16\textsuperscript{INK4A} (5-GTGAGCCTGCTGAGGAGGAG-3 and 5-CCTTCCATCTGGGATGTCTG-3), and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 5-AGCCACATCGCTCAGACAC-3 and 5-GGGGGATGTCTG-3). The resulting PCR products were subjected to 2% agarose gel electrophoresis, and the intensity of band was determined with the use of MultiGauge software (Fujiﬁlm). Quantitative real-time PCR was done using a StepOne Real-time PCR (Applied Biosystems) and the SYBR Green system. Data were normalized by the expression level of GAPDH in each sample and shown as the relative expression to those of parental cells.
Luciferase Reporter Assay for Activity of the p16INK4A Gene Promoter

SYO-1 and Fuji cells were transfected with both a firefly luciferase reporter plasmid for the promoter of the human p16INK4A gene and the Renilla luciferase plasmid pRL-TK (Promega) and analyzed as described (37). Data represent mean ± SD of experiments done in triplicate and subjected to one-way ANOVA followed by the comparison by Student’s t test. P values obtained from the tests are described in the figure legends.

Cell Cycle Analysis

Parental or Crk-silenced SYO-1 cells were fixed overnight at 4°C with 70% ethanol. The fixed cells were incubated in the dark for 60 min at room temperature with RNase (type I-A; Sigma) at 0.1 mg/mL and propidium iodide (Sigma) at 100 μg/mL, and the fluorescence of propidium iodide was then measured by flow cytometry with a FACSCanto instrument (BD). The percentage of cells in each phase of the cell cycle was determined with the use of FlowJo 8.0.1 software (BD).

Tumor Formation in Nude Mice

Nude mice were injected s.c. with parental or Crk-silenced Fuji cells as described previously (7). Total RNA was isolated from the resulting tumors and analyzed by RT-PCR as described above.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank S. Feller, M. Matsuda, and H. Hanafusa for expression plasmids and Y. Orba for technical assistance with flow cytometry.

References

Adaptor Protein Crk Induces Src-Dependent Activation of p38 MAPK in Regulation of Synovial Sarcoma Cell Proliferation

Takuya Watanabe, Masumi Tsuda, Shinya Tanaka, et al.

Mol Cancer Res  Published OnlineFirst September 8, 2009.

Updated version  Access the most recent version of this article at:
doi:10.1158/1541-7786.MCR-09-0064