Adaptor Molecule Crk Is Required for Sustained Phosphorylation of Grb2-Associated Binder 1 and Hepatocyte Growth Factor–Induced Cell Motility of Human Synovial Sarcoma Cell Lines

Takuya Watanabe,1,2 Masumi Tsuda,1 Yoshinori Makino,1 Shin Ichihara,1 Hirofumi Sawa,1 Akio Minami,2 Naoki Mochizuki,3 Kazuo Nagashima,1 and Shinya Tanaka 1

1Laboratory of Molecular and Cellular Pathology and 2Department of Orthopaedic Surgery, Hokkaido University Graduate School of Medicine, Sapporo, Japan and 3National Cardiovascular Center Research Institute, Osaka, Japan

Abstract

Activation of the c-Met receptor tyrosine kinase through its ligand, hepatocyte growth factor (HGF), promotes mitogenic, motogenic, and morphogenic cellular responses. Aberrant HGF/c-Met signaling has been strongly implicated in tumor cell invasion and metastasis. Both HGF and its receptor c-Met have been shown to be overexpressed in human synovial sarcoma, which often metastasizes to the lung; however, little is known about HGF-mediated biological effects in this sarcoma. Here, we provide evidence that Crk adaptor protein is required for the sustained phosphorylation of c-Met-docking protein Grb2-associated binder 1 (Gab1) in response to HGF, leading to the enhanced cell motility of human synovial sarcoma cell lines SYO-1, HS-SY-II, and Fuji. HGF stimulation induced the sustained phosphorylation on Y307 of Gab1 where Crk was recruited. Crk knockdown by RNA interference disturbed this HGF-induced tyrosine phosphorylation of Gab1. By mutational analysis, we identified that Src homology 2 domain of Crk is indispensable for the induction of the phosphorylation on multiple Tyr-X-X-Pro motifs containing Y307 in Gab1. HGF remarkably stimulated cell motility and scattering of synovial sarcoma cell lines, consistent with the prominent activation of Rac1, extreme filopodia formation, and membrane ruffling.

Introduction

Cell motility is essential for a variety of biological and physiologic processes, including embryonic development, wound healing, angiogenesis, and organogenesis, whereas an aberrant motility is observed in several human malignant tumors that could be linked to promoting tumor cell invasion and metastasis correlating with poor prognosis. Several growth factors have been shown to stimulate cell motility, and one of the most potent inducers is hepatocyte growth factor (HGF). HGF is a multifunctional factor that influences cell proliferation, migration, adhesion, and organ and tissue development through the activation of its receptor tyrosine kinase c-Met (1). Above all, HGF has also been known as scattering factor (2). HGF triggers autophosphorylation on Y1234 and Y1235 in the catalytic domain of c-Met (3). Moreover, two tyrosine residues (Y1349 and Y1356) of c-Met are also autophosphorylated on HGF stimulation (4), which provide a multisubstrate binding site for several Src homology 2 (SH2) domain–containing cytoplasmic effectors. In addition to the several SH2 domain–containing molecules, Grb2-associated binder 1 (Gab1) also interacts with the activated c-Met directly through its Met-binding sequences (5) or indirectly via the adaptor protein Grb2 (6, 7).

Gab1 is a member of docking protein and plays a role to integrate the signals of downstream of c-Met. Gab1 is phosphorylated on multiple tyrosine phosphorylation sites on c-Met kinase stimulation that are required for recruiting a variety of SH2 domain–containing transducers, including the p85 subunit.
of phosphatidylinositol 3-kinase, phospholipase C\(_\gamma1\), tyrosine phosphatase SHP-2, SHIP, and adaptor protein Shc, CrkII, and Crk-like (CrkL; refs. 6, 8-14). Especically, Gab1 possesses six Tyr-X-X-Pro (YXXP) motifs those are potential binding sites for adaptor protein CrkII and CrkL.

Crk has been shown to mediate a variety of biological responses involved in proliferation, cell differentiation, and migration. Crk was originally identified as an avian sarcoma virus CT10 encoding protein, v-Crk (15). Mammalian homologue CrkII is composed of a SH2 domain and two SH3 domains, and its alternative splicing form CrkI contains the SH2 domain and only one SH3 domain (16). The Crk SH2 domain binds several tyrosine-phosphorylated proteins, including p130\(^{Cas}\), paxillin, Cbl, and Gab1. The SH3 domain of Crk has been shown to interact with guanine nucleotide exchange factors (17), including Dock180, which activates Rac1 and regulates cell motility (18, 19), whereas the activation of Rap1 through the another Crk SH3 domain target C3G (20, 21) is involved in cell adhesion (22).

Recent studies show the involvement of Crk in tumorigenesis and progression. Human CrkI can induce transformation in rat fibroblasts (16), and Crk has been known to be overexpressed in various human cancers, including brain tumors and lung cancers (23-25). However, the precise role for Crk in human cancer is still under investigation.

Abnormal HGF/c-Met signaling has been reported to be strongly implicated in the development and progression of a variety of human tumors. The amplification and point mutations in kinase domain of c-Met have been found in gastric carcinomas and gliomas (26, 27) and in hereditary and sporadic papillary renal cell carcinomas (28), respectively. Moreover, the overexpression of HGF and/or c-Met is associated with a poor prognosis of breast cancer (29) and multiple myeloma (30). Transgenic mice overexpressing HGF develop tumors with metastatic lesions (31). Synovial sarcoma is a malignant soft tissue tumor characterized by the oncogenic chimeric transcript SYT-SSX arising from chromosomal translocation t(X,18) (32, 33).

FIGURE 1. HGF-induced tyrosine phosphorylation of c-Met and Gab1 in human synovial sarcoma cell lines. A. Expression levels of c-Met and Gab1 in human synovial sarcoma cell lines SYO-1, HS-SY-II, and Fuji were examined by immunoblotting. B. HGF-induced tyrosine phosphorylation of c-Met and Gab1. SYO-1, HS-SY-II, and Fuji cells were treated with or without 50 ng/mL HGF for 30 minutes. The cell lysates were immunoprecipitated with or without anti-Met or anti-Gab1 antibody and subsequently probed with the indicated antibody. C. HGF-induced interaction of c-Met and Gab1. HGF treatment was done as described in B. The cell lysate was immunoprecipitated using anti-Gab1 antibody, and c-Met bound to Gab1 was detected by immunoblotting. D. HGF-independent tyrosine phosphorylation of p130\(^{Cas}\) and paxillin. With or without HGF treatment, p130\(^{Cas}\) and paxillin were immunoprecipitated by each antibody and probed with anti-phosphotyrosine antibody.

Results

HGF-Induced Tyrosine Phosphorylation of c-Met and Gab1 in Human Synovial Sarcoma Cell Lines

Human synovial sarcoma has been shown to overexpress both HGF and its receptor c-Met; therefore, we initially examined the expression levels of c-Met protein in three independent human synovial sarcoma cell lines, SYO-1, HS-SY-II, and Fuji. c-Met was found in all cell lines,
particularly higher level in SYO-1 cells (Fig. 1A), consistent with the previous studies that the epithelial components in biphasic synovial sarcoma highly express c-Met (39). Following HGF stimulation, the tyrosine-phosphorylated form of c-Met became detectable in all cell lines (Fig. 1B, top), particularly stronger in SYO-1 cells. Phosphorylation site-specific antibody identified the autophosphorylation on Y1234 and Y1235 in the catalytic domain of c-Met (Fig. 1B) that have been shown to be essential for the kinase activity of c-Met. Moreover, the phosphorylation on Y1349 of c-Met was also induced on HGF stimulation (Fig. 1B), which is the direct binding site for Gab1. Gab1 was expressed in all three cell lines (Fig. 1A), and HGF-dependent association of c-Met and Gab1 was detected by immunoprecipitation analysis (Fig. 1C). In addition, the tyrosine-phosphorylated forms of Gab1 became also detectable on HGF stimulation; indeed, Y307 residue, which is one of six Crk-binding consensus YXXP motifs, was identified as one of the phosphorylation sites (Fig. 1B). The results of immunoblotting of HGF-dependent phosphorylation of Met and Gab1 in all three synovial sarcoma cell lines by using total cell lysates were shown in Supplementary Data 1A. In contrast, HGF stimulation did not significantly enhance the tyrosine phosphorylation of p130Cas and paxillin, except for slightly enhanced phosphor-}

Crk-SH2 Domain–Dependent Regulation of Tyrosine Phosphorylation of Gab1

As expected from the result of HGF-induced phosphorylation on Y307 of Gab1 (Fig. 1B), HGF treatment induced the recruitment of Crk to Gab1 (Fig. 2A). To clarify the precise binding mechanism between Gab1 and Crk, the deletion mutant of Gab1 and the point mutants of CrkII were transiently overexpressed in 293T cells (Fig. 2B). We initially found that the exogenously expressed CrkII with functional SH2 domain, such as CrkII-WT and CrkII-W169L, induced the prominent phosphorylation within several YXXP motifs of Gab1 (Fig. 2B). In contrast, CrkII lacking the function of SH2 as CrkII-R38V completely failed to induce this phosphorylation (Fig. 2B). In immunoprecipitation analysis, we found that anti-Gab1 antibody efficiently coprecipitated wild-type CrkII but not CrkII-R38V; furthermore, HGF stimulation enhanced the association of Crk and Gab1 (Fig. 2C). The endogenous expression levels of c-Met were previously confirmed to be almost equal between 293T and SYO-1 cells (data not shown). Thus, the mutational analyses of Gab1 and Crk indicate the crucial role of Crk on the stabilization of HGF-induced tyrosine phosphorylation of Gab1.

Establishment of Crk Knockdown Human Synovial Sarcoma Cell Lines

To clarify the significance of Crk-mediated phosphorylation of Gab1 in the biology of synovial sarcoma, we established Crk knockdown SYO-1, HS-SY-II, and Fuji cell lines by small interfering RNA technique. These cells were confirmed to contain substantial amount of endogenous Crk (data not shown) as reported in various human tumors. We succeeded in establishing several vector control and Crk knockdown cell lines in three cell lines. In Crk knockdown cells, the expression levels of CrkI and CrkII proteins were significantly suppressed (Fig. 3A), whereas the expression levels of CrkL as well as actin were constant (Fig. 3A).

Suppression of HGF-Dependent Sustained Phosphorylation of Gab1 in Crk Knockdown Synovial Sarcoma Cell Lines

To examine the involvement of Crk on the stabilized phosphorylation of Gab1, we analyzed the time-dependent
alteration on phosphorylation of c-Met and Gab1 following HGF stimulation in wild-type SYO-1 cells and its Crk knockdown cells. In parental SYO-1 cells, c-Met protein was significantly degraded within 3 hours after HGF stimulation (Fig. 3B). The kinase activity of c-Met, characterized by the phosphorylation on Y1234 and Y1235 residues in its catalytic domain, reached a maximum level at 20 minutes after HGF stimulation and gradually decreased (Fig. 3B). HGF treatment also triggered remarkable phosphorylation on Y307 of Gab1 (Fig. 3B) that was sustained >2 hours after HGF stimulation, contrary to rapid decline of c-Met phosphorylation. The association of Crk to Gab1 was also sustained over 3 hours after HGF stimulation (Fig. 3B). On the other hand, we detected no significant alteration on the endogenous expression levels of c-Met and its down-regulations following HGF-stimulation between wild-type SYO-1 cells and its Crk knockdown cells (Fig. 3B). However, in Crk knockdown SYO-1 cells, tyrosine phosphorylation on Y307 of Gab1 started to decrease after reaching maximum level at 10 minutes after HGF stimulation, and this phosphorylation was undetectable within 1 hour (Fig. 3B and C). These data suggest that the elimination of Crk caused the reduced phosphorylation of Gab1 and its rapid down-modulation (Fig. 3C). Indeed, in Crk-depleted SYO-1 cells, the phosphorylation levels of Gab1 at 30 and 60 minutes following HGF stimulation reduced to 0.75 and 0.6, respectively, relative to that of its wild-type cells (Fig. 3C).

In established Crk knockdown cells, the expression levels of Crk-SH3 targets, such as Dock180 and C3G, were also analyzed. SYO-1 and HS-SY-II cell lines possess almost equal SH3 targets between control and Crk knockdown cells, whereas in Fuji cells the levels of Dock180 and C3G seemed to be weakened by Crk elimination (Fig. 3D).

Analysis of Rac1 Activity in Crk Knockdown Cells

Rac1 is one of the Rho family small GTPases that has been reported to regulate the cytoskeletal reorganization downstream of Crk/Dock180 association. Therefore, to identify the effect of Crk in HGF-induced cytoskeletal reorganization, we examined the activity of Rac1 in wild-type and Crk knockdown synovial sarcoma cell lines. HGF induced prominent activation of Rac1 in all parental cells. Especially, the biphasic activation of Rac1 within 90 minutes after HGF stimulation was shown with peak at 10 and 30 minutes in SYO-1 cells and at 5 and 30 minutes in HS-SY-II cells (Fig. 4, top left and middle). This biphasic activation of Rac1 was remarkable in Fuji cells that exhibited 4.0-fold increase at 20 minutes after HGF stimulation, reduction at 45 minutes, and a secondary 3.7-fold activation at 60 minutes (Fig. 4, bottom left). In contrast, HGF-mediated slight increase of Rac1 activity was detected in Crk knockdown SYO-1 cells (Fig. 4, top right). Furthermore, even mild decrease of Rac1 activity was observed with HGF treatment in Crk knockdown HS-SY-II and Fuji cells (Fig. 4, middle and bottom right).

To identify the exact subcellular localization and its extent of HGF-induced Rac1 activation in individual cells, we employed fluorescent resonance energy transfer (FRET)–based time-lapse microscopy. After transient transfection of established Crk knockdown synovial sarcoma cells.

A.

To identify the exact subcellular localization and its extent of HGF-induced Rac1 activation in individual cells, we employed fluorescent resonance energy transfer (FRET)–based time-lapse microscopy. After transient transfection of

![Image](image-url)
the probe for monitoring Rac1 activity (Fig. 5A), synovial sarcoma cell lines were treated with 50 ng/mL HGF. Positive signals indicating the activation of Rac1 were observed in all parental synovial sarcoma cell lines as red color, particularly at the cellular membrane of SYO-1 cells after HGF stimulation (Fig. 5B). In parental Fuji cells, positive signals were diffusely observed in the cytoplasm without HGF (Fig. 5C, a). On HGF stimulation, the careful observation showed the additional activation of Rac1 as spots around the cytoplasmic membrane (Fig. 5C, c, arrow) followed by subsequent cytoplasmic protrusion (Fig. 5C, d, square), leading to the dynamic morphologic change (Fig. 5C, e and f). In contrast, the activity of Rac1 was reduced by HGF stimulation in Crk knockdown Fuji cells (Fig. 5C, g-l), correlating with the result of the pull-down assay (Fig. 4).

**Crk-Dependent Cytoskeletal Reorganization on HGF Stimulation**

We proceeded to observe the appearances of actin cytoskeleton of synovial sarcoma cell lines to examine the effects of Rac1 activation involved in cytoskeletal reorganization. In both parental SYO-1 and HS-SY-II cells, HGF stimulation attenuated the formation of actin stress fibers; instead, the extreme prolonged and the numerous filopodia formations were produced in SYO-1 cells (Fig. 6A, d, arrows) and HS-SY-II cells (Fig. 6A, e, arrow), respectively. In Fuji cells, HGF induced the dynamic morphologic changes with filopodia formation at its leading edge (Fig. 6A, f, arrows). The prominent membrane ruffles were also detected in all cells on HGF stimulation (Fig. 6A, d-f, arrowheads). On the other hand, in Crk knockdown cells, actin was visualized by anti-actin antibody that more clearly delineated actin network compared with phalloidin recognizing...
only F-actin. In Crk-depleted Fuji cells, actin fibers were disorganized with random direction (Fig. 6B, b) and speckled pattern (Fig. 6B, c). To prove these aberrant organizations of actin were not caused by artificial error in the process of immunostaining, actin organization in the living cells were examined by monitoring the exogenously expressed green fluorescent protein–fused actin. Indeed, we confirmed the similar disorganized actin fibers and the speckled pattern in Crk knockdown Fuji cells (Fig. 6B, e and f). HGF treatment did not induce any alterations on these actin disorganizations (data not shown).

Analysis of Cell Motility and Scattering in Crk Knockdown Cells

Because HGF-dependent enhancement on the invasion was observed in all three synovial sarcoma cell lines (Supplementary Data 1B), we further did the wound-healing assays to analyze the effects of Crk elimination on HGF-induced cell motility. In the absence of HGF, Crk knockdown Fuji cells exhibited ~40% and 50% reduction of motility relative to control cells at 24 and 48 hours after scratching, respectively (Fig. 7A, left). HGF stimulation showed 1.4-fold increase on cell motility in both parental and control cells at 48 hours after scratching. However, Crk knockdown cells did not indicate any HGF-dependent enhancement on cell motility (Fig. 7A, right).

Furthermore, the time-lapse microscopic observation showed significant cell scattering in parental Fuji cells on HGF stimulation (Fig. 7B, a and b; Supplementary Data 2). Crk knockdown cells displayed flatter cell morphology compared with parental cells and could not dissociate from cell assembly even on HGF stimulation that resulted in showing no significant cell movement (Fig. 7B, c and d; Supplementary Data 3).

Suppression of Massive Tumor Formation by Crk Depletion in Nude Mice

To examine the significance of Crk on tumorigenic and/or metastatic phenotypes of synovial sarcoma in vivo, we have s.c. injected wild-type, empty, or Crk knockdown Fuji cells on the back of nude mice. One month after injection, wild-type and empty cells developed the tumor masses sized of 20.0 and 14.6 mm, respectively, on the average diameter (Fig. 8A). The microscopic analysis showed that newly formed tumors exhibited the typical morphologic features as synovial

![FIGURE 5. Analysis of Rac activity in Crk knockdown cells by FRET.](image)
sarcoma with the prominent mitosis (Fig. 8B, arrowheads) and the invaded cell growth into the peripheral tissues. Contrary, no tumor mass was formed in mice injected with Crk knockdown Fuji cells (Fig. 8A). In its barely formed tumors, we hardly detected the images of condensed chromatins in contrast to that in tumors composed of wild-type Fuji cells (Fig. 8B).

Discussion

The adaptor protein Crk is involved in cellular transformation, and currently, there are several reports that the overexpression of Crk correlates with aggressiveness of various human cancers (23-25). Crk also has been known to play a role in HGF/c-Met-mediated biological responses involved in morphogenesis, epithelial-mesenchymal transition, cell spreading, breakdown of epithelial adherence junction, and c-Jun NH2-terminal kinase activation for cell transformation (12, 40, 41). The overexpression of both HGF and c-Met has been shown in human synovial sarcoma (34-36), which possesses aggressive features, such as invasive growth into soft tissue around the knee joint and frequent metastasis to the lung (42). In this study, by the analysis of Crk knockdown synovial sarcoma cell lines, we found the crucial role of Crk in HGF/c-Met-mediated cell motility of human synovial sarcoma cell lines through the sustained phosphorylation of Gab1.

Gab1 is phosphorylated for a prolonged period of time (>60 minutes) on HGF stimulation in contrast to a transient phosphorylation in response to epidermal growth factor (43). Sustained phosphorylation of Gab1 is suggested to be required for the HGF-mediated responses. We also found that HGF stimulation induced the sustained phosphorylation (>3 hours) on Y307 residue of Gab1, which is the potential binding site for Crk, correlated with the recruitment of Crk to Gab1 (Fig. 3B).

The sustained phosphorylation of Gab1 could be due to various mechanisms, including the kinetics of kinase activation (44, 45), half-life of the ligand-receptor complex at the cell surface (46, 47), and differential activation of phosphotyrosine phosphatases, such as SHPTP-2 (48, 49). In addition, we hypothesized that the stable complexes of Crk with Gab1 via its SH2 domain may induce the sustained phosphorylation of Gab1 probably by protecting its phosphotyrosines from phosphatases, such as SHPTP-2. In fact, overexpression of Crk-SH2 domain could induce the prominent phosphorylation on multiple YXXP motifs of Gab1 in HGF-independent manner (Fig. 2B). In addition, the phosphorylation of Gab1 evoked by HGF in parental SYO-1 cells was sustained >3 hours although c-Met was significantly degraded probably by the ubiquitin-proteasome pathway (Fig. 3B; refs. 50-52). In agreement with our hypothesis, Crk knockdown by RNA interference reduced the duration of HGF-induced phosphorylation on Y307 residue of Gab1 in synovial sarcoma cell lines (Fig. 3B).

Y307 residue of Gab1, also known as the binding site for phospholipase Cγ, can induce HGF-mediated branching tubulogenesis but not cell motility or scattering (43). Because the endogenous expression levels of CrkI and CrkII proteins were elevated in synovial sarcoma cell lines (data not shown), the sustained complexes of Crk to Gab1 following HGF stimulation may activate specific signaling pathway controlling cell motility and scattering of this sarcoma.

As phosphatidylinositol 3-kinase has been reported to regulate Rac1 activity through the several guanine nucleotide exchange factors, such as SWAP70 and P-REX2 downstream of Gab1 (53-55), and Yav and Tiam-1 are also known to mediate Rac1 activity involved in transformation (56, 57), we initially speculated that the implication of Crk in HGF-mediated Rac1 activation may be partial. However, the significant suppression of HGF-dependent activation of Rac1, cell motility, and scattering was shown in Crk knockdown cells, which may suggest the crucial role for Crk in HGF-evoked activation of Rac1 and following cell motility in human synovial sarcoma cells.
On HGF stimulation, a biphasic activation of Rac1 was observed in synovial sarcoma cells (Fig. 4, left) in spite of constant phosphorylation of Gab1. We hypothesized that the differential activation of Rac1 in separate cell compartments may be involved in this biphasic activation of Rac1 as reported about Ras, which is activated in both the cytoplasmic membrane and the endomembranes of endoplasmic reticulum and Golgi (58). FRET-based time-lapse analysis was employed to examine this point; however, we were unable to identify the exact subcellular compartments arising the biphasic Rac1 activation because of its technical limitation. In our further analysis, the monitoring of the exogenously expressed red fluorescent protein–fused Dock180 in SYO-1 cells did not show HGF-mediated translocation of Dock180 to endoplasmic reticulum, whereas we could find the induction of Dock180 to the cell surface at 10 minutes after HGF stimulation, subsequently in all regions of newly formed filopodia at 30 minutes (Supplementary Data 1C). Furthermore, glutathione S-transferase pull-down assay in buffer containing CHAPS, which favors the release of the protein out of endoplasmic reticulum and Golgi, seemed to exhibit no effects on Rac1 activation in endoplasmic reticulum and Golgi (Supplementary Data 1D). Alternatively, the activation of Rac-GAP, such as p21-activated kinase, or GC-GAP may be involved in the biphasic activation of Rac (59, 60). The precise mechanisms underlying the biphasic activation of Rac1 in synovial sarcoma cells should be continued the investigation.

On the other hand, in Crk knockdown cells, we observed a decline of Rac1 activity after HGF stimulation (Fig. 4, right). This decreased activity of Rac1 may suggest that HGF provoked the activation of negative regulator for Rac1 activity, such as GC-GAP, by Crk-independent signaling mechanism. The activation of such negative signal in the absence of a Crk-dependent positive signal may induce down-regulation of Rac1 activity.

Crk-depleted cells failed to induce HGF-dependent membrane ruffling as observed in parental synovial sarcoma cells, indicating that the functional disruption of Crk/Dock180/Rac1 signaling pathway impairs HGF-mediated cytoskeletal reorganization in synovial sarcoma cells. Rac1 and Cdc42 are known to cooperatively act for the spreading of Madin-Darby canine kidney epithelial cells stimulated with HGF (59). Because synovial sarcoma cells could induce filopodia formation on HGF stimulation (Fig. 6A, arrows), Cdc42 also may contribute to the remodeling of the actin cytoskeleton in this sarcoma. Furthermore, Crk knockdown cells remarkably disrupted the organization of actin cytoskeleton as represented as random direction of actin fibers (Fig. 6B, b and e) and speckled pattern (Fig. 6B, c and f), which seemed to be similar to the disorganization of actin network on the Rho inhibitor C3 or its downstream ROCK inhibitor Y27632 (data not shown). These results may suggest the requirement of Crk on the activation of Rho in synovial sarcoma cells (61), although we hardly detect Crk-dependent alternations on the activation of Rho by glutathione S-transferase pull-down assay probably due to its limited sensitivity (data not shown). Thus, Crk may comprehensively contribute to the regulation of Rho family, including Rac1, Cdc42, and Rho.

C3G, other target for Crk, is known to regulate cell adhesion through the activation of Rap1, but no significant change of the cell attachment on culture dishes was observed in these Crk knockdown sarcoma cell lines (data not shown). Previous study has shown that Gab1 association with CrkL correlates with c-Met-evoked activation of the Rap1 and decreased adhesion of HEK293 cells (11). Because the expression of CrkL is retained in Crk knockdown cells (Fig. 3A), CrkL may play a dominant role in cell attachment.

Wild-type and empty Fuji cells formed the tumor masses with the peripheral invasion in nude mice. However, the depletion of Crk remarkably inhibited the massive tumor growth. Consistent with these results, we further found that Crk-depleted cells exhibited the suppression of growth rate in vitro and the loss of colony formation in soft agar (data not shown). These results may suggest the crucial role of Crk on the tumorigenesis of synovial sarcoma in addition to its function as the regulator of cell motility and invasion.
Our study shows a requirement of Crk in HGF-mediated constitutive activation of Rac1 through the sustained coupling of Crk to Gab1 result in promoting cell motility and scattering of human synovial sarcoma cells. Thus, Crk might be an attractive therapeutic target for human synovial sarcoma.

**Materials and Methods**

**Cells**

Human synovial sarcoma cell lines SYO-1, HS-SY-II, and Fuji were established by A. Kawai (National Cancer Center, Tokyo, Japan; ref. 39), H. Sonobe (Kochi Medical University, Kochi, Japan), and T. Nojima (Kanazawa Medical University, Kanazawa, Japan), respectively. HS-SY-II cells genetically occur as a SYT-SSX1 fusion transcript, whereas SYO-1 and Fuji cells possess SYT-SSX2. In the original tumors, the typical monophasic morphologies were observed in HS-SY-II and Fuji cells, whereas SYO-1 cells exhibited the biphasic features composed of areas of the spindle cells and that of the epithelial cells arranged in glandular structures. SYO-1, HS-SY-II, and 293T human embryonic kidney cells were maintained in DMEM supplemented with 10% fetal bovine serum, and Fuji was maintained on type I collagen-coated dishes in RPMI 1640 with 10% fetal bovine serum.

**Antibodies**

The antibodies against phosphotyrosine (PY20 and RC20H), p130Cas, paxillin, Crk, and Rac1 were purchased from Transduction Laboratories (Lexington, KY). Anti-Gab1 antibody was from Upstate (Lake Placid, NY). Anti-Met (C-12), C3G (C19), Dock180 (H4), and Crk-L (C20) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies against phosphorylated Met (Y1234/Y1235) and (Y1349) and phosphorylated Gab1 (Y307) were purchased from Cell Signaling (Beverly, MA). Anti-actin antibody was from Chemicon International (Temecula, CA). Anti-Flag antibody (M2) was from Sigma (St. Louis, MO). The phalloidin-594 for actin staining was purchased from Molecular Probes (Eugene, OR).

**Plasmid**

cDNAs of CrkII-WT, SH2 mutant (R38V), and NH2-terminal SH3 mutant (W169L) were subcloned into pCXN2 expression vector with NH2-terminal Flag tag sequence (DYKDDDDK). pBat-Flag-Gab1 and pBat-Flag-Gab1 YXXP were generous gifts from S.M. Feller (Oxford University, Oxford, United Kingdom). pGEX-PAX2-RBD for pull-down assay of Rac was described previously (62). pEGFP-actin was purchased from Clontech (Mountain View, CA).

**Immunoprecipitation and Immunoblotting**

Cells were lysed with a lysis buffer [10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 0.5% NP40, 50 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L Na3VO4] for 10 minutes on ice and centrifuged at 15,000 rpm for 10 minutes at 4°C. Supernatants were incubated with the appropriate antibody at 4°C with gentle shaking overnight followed by incubation with protein A/G-Sepharose. After washing with lysis buffer, the immunocomplexes were subjected to SDS-PAGE, and separated proteins were transferred to a polyvinylidene difluoride filter (Immobilon, Millipore, Billerica, MA). A blocking buffer was used to reduce nonspecific signals (1% bovine serum albumin in TBS-Tween 20 for detecting phosphotyrosine, 5% skim milk in TBS-Tween 20 for other proteins) before incubation with primary antibodies followed by peroxidase-labeled secondary antibodies. Signals were detected by the enhanced chemiluminescence Western blotting reagent (Amersham Pharmacia Biotech, Piscataway, NJ) and quantified using a Luminol Image Analyzer (LAS1000, Fuji Film, Tokyo, Japan).
Establishment of Crk Knockdown Synovial Sarcoma Cell Lines

The plasmid containing small interfering RNAs for Crk (CrKI) was described previously (63). The CrkI correspond- ing to the bases 277 to 296 of human c-CrkII encoding sequences as 5'-GAGTTTGATCTGCGCTG-3' and 5'- CGGCTAAGTTCACTC-3' were subeloned into SalI and KpnI sites of the pSUPER (suppression of endogenous RNA) vector (64) and transinfected into synovial sarcoma cell lines by Fugene 6 transfection reagent (Roche, Indianapolis, IN). Following selection with 0.5 μg/mL puromycin (Sigma), expression levels of Crk were confirmed by immunoblotting.

Analysis of the Actin Cytoskeleton

Cells were cultured on eight-chamber slides (Nunc, Naperville, IL). With or without 50 ng/mL HGFTreatment for 60 minutes, cells were fixed with 3.5% formaldehyde in PBS, After permeabilization with 0.1% Triton X-100 in PBS for four minutes at room temperature and blocking with 1% bovine serum albumin for 20 minutes at room temperature, actin was stained by phalloidin-594 (1:40 dilution) in PBS for 1 hour at room temperature in the dark and observed using a confocal laser scanning microscopy (FV-300, Olympus, Tokyo, Japan). In addition, green fluorescent protein–labeled actin cytoskeleton transfected in wild-type and Crk knock- down Fuji was observed using a fluorescent microscopy equipped with MetaMorph software (Universal Imaging, Downingtown, PA).

Pull-down Assay for Rac Activity

The GTP-bound form of Rac was detected as described previously (62). Synovial sarcoma parental cells and CrkI cells treated with or without HGFTreatment were lysed with lysis buffer [1% NP40, 25 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 10% glycerol, 1 mmol/L EDTA, 10 mmol/L MgCl2, 1 μg/mL aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride]. Cell lysates were centrifuged at 12,000 rpm at 4°C for 1 minute. The supernatant was incubated with 10 μg purified glutathione S-transferase-PK2-RBD followed by incubation with glutathi-one-Sepharose beads for 1 hour at 4°C while rotating. The beads were washed thrice with lysis buffer, and the precipitates were subjected to SDS-PAGE. After transfer to filter, the GTP-bound Rac was detected by mouse monoclonal anti-Rac1 antibody.

FRET-Based Time-Lapse Analysis of Rac Activity

The FRET probe for monitoring Rac activity, pRaiuich-Rac-CAAX, was a generous gift from M. Matsuda (Osaka University, Osaka, Japan; ref. 65). The plasmid was transfected into cell lines and incubated at 37°C for 36 hours. After treatment with HGF (50 ng/mL), FRET reaction in single cells was analyzed using MetaMorph software.

Wound-Healing Assay

The wound-healing assay was done as described previously (66). Briefly, 48 hours after plating on culture dishes, the cells were scraped off/wounded using a yellow tip. After 24 and 48 hours, movement of the cells was measured.

Analysis of Scattering

The scattering of cells followed by 50 ng/mL HGFTreatment was observed by phase-contrast microscopy using the Meta- Morph software. Images were saved at 5-minute intervals for a period of 24 hours.

In vivo Tumor Formation Assay in Nude Mice

Wild-type Fuji cells, empty-plasmid transfected cells (empty cells), and its Crk-depleted cells were employed to examine the potential on the tumorigenesis and the invasive growth into nude mice. Each 1 × 107 cells in PBS were s.c. injected into the 6-week-old female nude mice, BALB/c Jelnu nu/nu (Clea Japan, Inc., Tokyo, Japan). Three mice each for wild-type and empty cells and six mice for Crk-depleted cells were employed. One month after cell inoculation, the mice were euthanized, and the diameters of the formed tumors were determined. The histologic features were identified on H&E staining.

Acknowledgments

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References


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