Cooperative Roles of Fyn and Cortactin in Cell Migration of Metastatic Murine Melanoma*

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Src family kinases are major regulators of various integrin-mediated biological processes, although their functional roles and substrates in cancer metastasis are unknown. We explored the roles of Src family tyrosine kinases in cell migration and the spread of K-1735 murine melanoma cell lines with low or high metastatic potential. Corresponding to elevated cell motility and spreading ability, Fyn was selectively activated among Src family kinases, and the cell motility was blocked by an inhibitor of Src family kinases. Significant tyrosine phosphorylation of cortactin, stable complex formation between activated Fyn and cortactin, and co-localization of cortactin with Fyn at cell membranes were all observed only in cells with high metastatic potential. Both integrin-mediated Fyn activation and hyperphosphorylation of cortactin were observed 2-5 h after stimulation in highly metastatic cells, and they required de novo protein synthesis. We demonstrate that cortactin is a specific substrate and cooperative effector of Fyn in integrin-mediated signaling processes regulating metastatic potential.

Malignant tumors are thought to contain subpopulations of cells with differential metastatic capabilities (1). Processes of tumor metastasis consist of multiple steps linked together, including invasion, detachment, intravasation, circulation, adhesion, extravasation, and growth in distant organs (2). Cell locomotion and spreading are key functions of the cells required in most of these processes. Analysis of differences in expression and modification of signaling molecules associated with cell migration and spreading among tumor sublines with different metastatic potentials in a tumor is expected to provide precise information for understanding the molecular mechanisms underlying the development of cancer metastasis.

There are studies demonstrating that Src family tyrosine kinases play essential roles in the signaling of integrin-mediated biological processes such as actin organization and cell migration (3–7). In addition, recent reports show that Src is highly activated in colon cancers, particularly in those metastatic to the liver (8). Involvement of Src kinase during metastatic spread of carcinoma cells in NBT-II rat carcinoma cell lines has also been suggested (5). Fyn has been suggested a factor governing the metastatic potential of tumors including murine methylcholanthrene-induced fibrosarcoma cells (9), although the precise mechanism is unknown.

Increasing numbers of studies have shown that Src family kinases function through collaboration with their substrates, such as FAK, cortactin, p130Cas (a Crk-associated substrate), and paxillin in cytoskeleton organization and cell migration. FAK is activated upon cell binding to extracellular matrix proteins and forms transient signaling complexes with Src family kinases (10). Cortactin plays essential roles in cortical actin cytoskeleton organization, primarily affecting cell motility and invasion (11–13). A docking protein, p130Cas, plays essential roles in cell attachment and migration when it is tyrosine-phosphorylated by Src family kinases (6). Functional roles of paxillin in integrin-mediated signaling have been implicated by tyrosine phosphorylation of paxillin following integrin-dependent cell adhesion to extracellular matrix proteins, which is in part attributed to Src family kinases (14). To obtain further information on their involvements in the progression of metastasis, appropriate biological models showing different metastatic potentials are required.

A widely used model for studying the molecular mechanisms underlying the progression of metastasis is a series of cell lines derived from K-1735 murine melanoma, which contains heterogeneous clones with multiple metastatic diversities (15). The primary K-1735 melanoma that arose in an inbred C3H/HeN murine mammary tumor virus-negative mouse was transplanted once into an immunosuppressed recipient and then established in culture. Randomly chosen clones C10 and C19 were classified as nonmetastatic or low metastatic; M2 and X21 were highly metastatic and produce tumor foci in lungs of syngenic mice (15). In this study, we investigated the roles of Src family tyrosine kinases in cell migration and the spread of these cell lines. Up-regulation of Fyn kinase activity and enhanced tyrosine phosphorylation of cortactin were identified in highly metastatic cells, which also showed elevated cell motility and spreading ability. A new mode of integrin-mediated activation of Fyn was also observed in highly metastatic cells. These results indicate a novel role of the Fyn-cortactin pathway required for the regulation of cell motility and metastatic potential during the progression of metastasis.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture—K-1735-derived mouse melanoma cell lines were donated by Dr. I. J. Fidler. Clones C-10 and C-19 are classified as nonmetastatic or low metastatic. Parental clones M-2 and X21 are highly metastatic and produce tumor foci in lungs of syngenic mice (15).
3Y1-Crk is an isolated clone of rat 3Y1 cells transfected with v-Crk cDNA of an avian sarcoma virus CT10 inserted in expression vector pMV-7. 3Y1-Vec is an isolated clone of rat 3Y1 cells transfected with expression vector pMV-7 (16). All tumor cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Sigma) supplemented with 10% fetal calf serum (FCS; Sigma) and grown in the presence of penicillin and streptomycin (Sigma) at 37 °C.

Antibodies and Reagents—Polyclonal antibodies against Src family tyrosine kinase (Src-2), Src (Src-N-16), or Fyn (Fyn3) were obtained from Santa Cruz Biotechnology. Anti-phosphotyrosine antibody 4G10, monoclonal antibody against cortactin (clone 4F11) were obtained from Upstate Biotechnology, Inc. Monoclonal antibodies against Yes, Hck, or FAK were obtained from Transductions Laboratories. Monoclonal antibody against paxillin was obtained from Zymed Laboratories Inc. Polyclonal antibodies against Cas (Cas3 and Cas2) were used as described previously (16). Rhodamine-conjugated phalloidin was purchased from Molecular Probes, and fluorescein isothiocyanate-conjugated anti-mouse and rhodamine-conjugated anti-rabbit antibodies were obtained from Santa Cruz Biotechnology. Polylysine, fibronectin, and cycloheximide were purchased from Sigma. Src family kinases inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) and the structural analog 4-amino-7-phenylpyrazolo[3,4-d]pyrimidine (PP3) were obtained from Calbiochem-Novabiochem Ltd.

Cell Lysis, Immunoblotting, and Immunoprecipitation—Protein extraction and Western blotting analysis were performed as described (6). Briefly, the cells were lysed in 1% Triton X-100 buffer (see below). Total cell proteins in lysates were standardized prior to use.

Cell Lysis, Immunoblotting, and Immunoprecipitation—Protein extraction and Western blotting analysis were performed as described (6). Briefly, the cells were lysed in 1% Triton X-100 buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM EGTA, 100 mM NaF, 1 mM Na₃VO₄, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethanesulfonyl), and insoluble material was removed by centrifugation. The protein aliquots were separated by SDS-PAGE and probed with 1:2000 diluted antibodies. For immunoprecipitation, 500 μg of protein was mixed with 1–2 μg of antibodies against cortactin, Src family (Src-2), Src (Src-N-16), Fyn, Cas3, paxillin, or FAK and incubated for 1 h on ice. Then samples were rotated with
protein A- or protein G-Sepharose beads (Sigma) for 1 h at 4 °C. The beads were washed four times with 1% Triton X-100 buffer and boiled in sample buffer (2% SDS, 0.1 M Tris-HCl, pH 6.8, 10% glycerol, 0.01% bromphenol blue, 0.1 M dithiothreitol) before being subjected to SDS-PAGE analysis.

Immunofluorescence—Immunofluorescence staining was performed
Overexpression and hyperphosphorylation of cortactin in cells with high metastatic potential. Cells plated on plastic culture dishes for 48 h were lysed in 1% Triton X-100 buffer as described under “Experimental Procedures.” Tyrosine phosphorylation of cortactin, p130Cas, paxillin, and FAK in equal portions of cell lysates were analyzed by immunoprecipitation (IP) using antibody against cortactin (UBI), p130Cas, paxillin (Zymed Laboratories Inc.), or FAK (Transduction Laboratories). The immunoprecipitates were subjected to immunoblotting analysis by anti-phosphotyrosine antibody 4G10. As a control, quantity of cortactin, p130Cas, paxillin, or FAK in equal amount of whole cell lysates were also analyzed by immunoblotting (IB). A, expression and tyrosine phosphorylation of cortactin are up-regulated in highly metastatic cells X21 and M2. B, expression and tyrosine phosphorylation of p130Cas, paxillin, and FAK in melanoma cells. C, PP2 treatment impaired tyrosine phosphorylation of cortactin in highly metastatic cell lines X21 (upper panel) and M2 (lower panel). The cells cultured in DMEM with 10% FCS were washed by DMEM and then treated by PP2 (10 μM), Me2SO (10 μM), or PP3 (10 μM) in DMEM for 15 min before being lysed in 1% Triton X-100 buffer. Cortactin in equal portions of cell lysates was isolated by immunoprecipitation and subjected to immunoblotting by anti-phosphotyrosine antibody 4G10.

Specific association between cortactin and Fyn in highly metastatic melanoma cells. A, physical association between cortactin and Fyn. Equal amounts of immunoprecipitates (IP) by antibody against Fyn (Fyn3) (Santa Cruz Biotechnology) were subjected to immunoblotting (IB) by anti-cortactin antibody. The expression of Fyn was indicated by immunoblotting analysis of equal amount of whole cell lysates using antibody against Fyn (Fyn3). Enhanced association between cortactin and Fyn in X21 and M2 were observed. B and C, tyrosine-phosphorylated proteins associated with Fyn (B) or Src (C) were analyzed by immunoblotting equal portion of immunoprecipitations against Fyn or Src (Src-N-16, Santa Cruz Biotechnology) with anti-phosphotyrosine antibody 4G10 (UBI). Tyrosine phosphorylation of Fyn-associated protein with a size of 85 kDa (indicated by closed triangle) in X21 and M2 and tyrosine phosphorylation of Src-associated protein in M2 with a size of 80 kDa (indicated by open triangle) were observed.
Cell Migration Assay—Cell motility of 3Y1-Crk fibroblasts was performed as described previously (6). 6 × 10^4 cells were plated onto 12-mm circle cover glasses (Fisher), which were placed in each well of a 24-well plate and allowed to grow for 24 h in DMEM with 10% FCS at 37 °C with 5% CO₂. The number of cells on the lower side of the filter was counted from at least eight fields (error bars show the standard deviation). The results presented here are representative mean values of experiments performed three times. B, tyrosine-phosphorylated proteins p130Cas and P85 were associated with Fyn in 3Y1-Crk fibroblasts. C, elevated tyrosine phosphorylation of cortactin in 3Y1-Crk fibroblasts. Fyn-associated tyrosine-phosphorylated protein and phosphotyrosine of cortactin in 3Y1-Crk fibroblasts were determined by immunoblotting equal portion of immunoprecipitations (IP) against Fyn or cortactin (UBI) with anti-phosphotyrosine antibody 4G10 (UBI). The quantity of Fyn or cortactin in an equal amount of whole cell lysates was also analyzed by immunoblotting (IB).
each microscope field. The same experiment was repeated at least three times.

**Immune Complex Kinase Assay**—For immune complex assay, each member of Src family kinases in cell lysates containing 500 μg of proteins was first immunoprecipitated by antibody against Fyn (UBI), Src family (Src2), Src (Src-N-16) (Santa Cruz Biotechnology), Yes, or Hck (Transduction Laboratories). Then immunoprecipitates were consequently washed using 1% Triton buffer and kinase buffer (50 mM Tris, pH 7.4, 50 mM NaCl, 10 mM MgCl₂, 10 mM MnCl₂) three times, respectively. To each sample, 1 μl of exogenous synthetic polypeptides poly-[Glu-Tyr] was added as exogenous substrate. Kinase reaction was performed in 30 μl of kinase buffer with 5 μCi of [γ-³²P]ATP (ICN) at room temperature for 30 min. Kinase reactions were stopped by the addition of SDS-PAGE sample buffer (2% SDS, 0.1 M Tris, pH 6.8, 10% glycerol, 0.01% bromphenol blue, 0.1 M dithiothreitol). The samples were then subjected to SDS-PAGE analysis using 9% polyacrylamide gel. The gels were then dried and exposed to autoradiography.

**RESULTS**

**Highly Metastatic Marine Melanoma Cell Lines Exhibited Enhanced Motility and Spreading Ability on Fibronectin**—To clarify differences in cell properties that correspond to the diversity of metastatic potentials of K-1735 cell lines, we measured haptotactic cell migration ability toward FN using a Boyden chamber. As shown in Fig. 1A, about 140 cells/field of highly metastatic cells X21 and M2 migrated through the pores of the filter in 3 h toward FN. In contrast, for low metastatic cells C19 and C10, only about 70 cells/field of C19 and no more than 10 cells/field of C10 migrated through the pores toward FN, respectively. No cell locomotion was observed in the absence of FN in any of these cell lines, suggesting that these cells exhibit FN-dependent cell motility. Cell motility under the stimulation of integrin correlates with metastatic potentials.

To determine their differences in spreading abilities, time-dependent cell spreading was estimated by the percentage of flat cells in all cells at attachment after plating on FN-coated dishes (see “Experimental Procedures”). About 70% of X21 and 45% of M2 exhibited a flat shape with multiple protrusions 30 min after plating on FN. In contrast, about 20% of C19 and 17% of C10 were spread (Fig. 1B). After plating on FN for 60 min, the percentages of flat cells of X21 and M2 increased to about 98%, whereas there were less than 50% C19 and C10 showing a flat shape (Fig. 1B). These results demonstrate that cell spreading of X21 and M2 occurs much faster than that of C10 and C19. Both cell migration and cell spreading abilities were enhanced in highly metastatic cell lines.

**Analysis of Src Family Kinases Responsible for Enhanced Cell Motility of Melanoma Cells with High Metastatic Potential**—Roles of Src family tyrosine kinases in cell migration of...
X21 and M2 cells were investigated by the Boyden chamber in the presence of specific inhibitor of Src family kinases including Src, Fyn and Hck, PP2 (18). The inactive structural analog PP3 and Me2SO were used as negative controls. Treatment of PP2 significantly reduced the number of cells migrating through the pores in 3 h from 140 to about 30/field (Fig. 2), whereas negative controls Me2SO or PP3 did not show such an effect, showing that PP2 impairs cell motility of X21 and M2. These results demonstrate that some member(s) of Src family kinases play essential roles in cell migration of highly metastatic cells.

To characterize the member of the Src kinases responsible for the enhanced cell motility and spreading ability detected in highly metastatic cells M2 and X21, the kinase activities of Src, Fyn, Yes, and Hck were investigated using an in vitro immune complex kinase assay in the presence of exogenous synthetic polypeptides poly[Glu-Tyr] as substrates. No significant differences in kinase activities of Src and Yes were observed in any of the cell lines with different metastatic potentials (Fig. 3, C and D). In the case of Hck, the kinase activity was rather lower in X21 and M2 (Fig. 3E), whereas the activity of Fyn was particularly elevated in highly metastatic cells X21 and M2 compared with C10 and C19 (Fig. 3B). These results indicate that Fyn is a candidate Src family kinase responsible for high metastatic potential in X21 and M2 melanoma cells.

In the absence of exogenous substrate, a distinct 85-kDa protein was significantly phosphorylated by the immune complex kinase assay by pan-Src and Fyn in X21 and M2 (Fig. 3, F and G, closed triangles) but not in C10 or C19. This protein was not detected in protein complexes immunoprecipitated by Src (Src-N-16), Yes, or Hck (Fig. 3, H–J), demonstrating that this protein is specifically associated with Fyn in highly metastatic cell lines. An 80-kDa phosphorylated band was also immunoprecipitated by Src, Hck, or Yes only in M2 (Fig. 3, H–I, open triangles). Judging from differences in molecular size and substrate specificity, this protein is distinct from the 85-kDa protein observed in both X21 and M2.

Overexpressed and Hyperphosphorylated Cortactin Was Specifically Associated with Fyn in Highly Metastatic Cells—Cortactin, a protein of 80/85 kDa, acts as a potential linker between membrane-located receptors and cytoskeleton, primarily affecting cell motility and invasion (11–13). Because the molecular size of cortactin appeared close to the size of the protein associated with Fyn in X21 and M2 (Fig. 3G), we examined the expression and tyrosine phosphorylation of cortactin in these murine melanoma cells. As expected, tyrosine phosphorylation of cortactin was significantly elevated in X21 and M2 (Fig. 4A, lanes 2 and 4) compared with that of C10 and C19 (Fig. 4A, lanes 1 and 3). Expression levels of cortactin in X21 and M2 were also up-regulated (Fig. 4B, lanes 1 and 3). Tyrosine phosphorylation levels of cortactin were much reduced by Src family kinase inhibitor PP2 in X21 and M2 in contrast to PP3 (Fig. 4C), confirming that Src family kinases are responsible for tyrosine phosphorylation of cortactin in these cells. At the same time, we investigated the expression and tyrosine phosphorylation of other substrates of Src family kinases such as p130Cas, FAK, and paxillin. As shown in Fig. 4B, tyrosine phosphorylation of p130Cas was reduced in X21 and M2 compared with that in C10 and C19, and no significant differences
tyrosine phosphorylation were detected among these cells. Tyrosine phosphorylation and expression of paxillin in C10 were much lower, but they were at the same level in C19 as in X21 and M2. Cortactin was the only phosphotyrosine-containing protein showing significantly elevated phosphorylation levels in highly metastatic cell lines. Further study confirmed that cortactin was specifically associated with Fyn in X21 and M2. Cortactin was the only phosphotyrosine-containing protein showing significantly elevated phosphorylation levels in highly metastatic cell lines.

Further study confirmed that cortactin was specifically associated with Fyn in X21 and M2. It was observed that the amount of cortactin coupling with Fyn in X21 and M2 was significantly greater than that in C10 and C19 (Fig. 5A). Correspondingly, tyrosine phosphorylation of the protein with a size of 85 kDa associated with Fyn in X21 and M2 was also observed (Fig. 5B, lanes 2 and 4). In contrast, an 80-kDa tyrosine-phosphorylated protein associated with Src was only observed in M2 but not in X21 (Fig. 5C, lane 4). This M2-specific protein was later identified as a gag protein derived from murine mammary tumor virus by means of mass spectrometry analysis, which might be infected during the establishment of the M2 cell line by in vivo selection. There has been no report on tyrosine phosphorylation of this protein so far, and its roles on the metastatic potential of M2 are currently under investigation.

To provide additional evidence for essential roles of kinase-substrate cooperation between Fyn and cortactin, v-Crk transformed 3Y1 fibroblasts were used in which Fyn was reported to be activated by v-Crk (19). Following Fyn activation, elevated cell motility was observed in 3Y1-Crk cells compared with mock-transfected 3Y1-vec cells as shown in Fig. 6A. At the same time, tyrosine phosphorylation of cortactin was significantly enhanced in 3Y1-Crk fibroblasts (Fig. 6C), and the same molecular sizes of phosphoproteins were obvious among Fyn-associated phosphoproteins apart from p130Cas, which has

\[ ^2 \text{J. Huang, T. Asawa, T. Takato, and R. Sakai, unpublished data.} \]
been reported to be associated with Fyn (Fig. 6B). These results in fibroblasts also support the possibility that cortactin tyrosine phosphorylation is regulated by Fyn in relation to signaling of cell migration.

Subcellular Localization of Fyn and Cortactin in Melanoma Cell Lines—Because cortactin has been functionally identified as an actin-associated protein (20–22), we also investigated co-localizations of cortactin with actin in C10, C19, X21, and M2. First, different distribution patterns of actin filaments between low and highly metastatic cells were noticed on non-coated glass slides. Significantly increased amounts of actin filaments were found at cell membrane protrusions in highly metastatic cells X21 and M2 (Fig. 7A, panels c2 and d2). In contrast, actin filaments were widely (nonspecifically) observed in cell matrix in C10 and C19 (Fig. 7A, panels a2 and b2). It was also observed that overexpressed cortactin was localized in these cell membrane protrusions specific to X21 and M2 (Fig. 7A, panels c1, d1, c3, and d3), implying that cortactin is associated with signaling of actin cytoskeleton organization near the membrane in highly metastatic cells. Results of staining of Fyn and cortactin demonstrated that most of cortactin and Fyn were distributed at cell membrane protrusions in M2 and X21 (Fig. 7B, panels a and d, arrows), showing apparent co-localization of cortactin and Fyn at these structures.

To further investigate the role of cortactin in highly metastatic cells, we explored co-localization of cortactin with actin filaments in X21 and M2 under the stimulation of integrin. As shown in Fig. 8, cortactin was co-localized with actin filaments at lamellipodia or fillopodia in M2 or X21, which were formed under the stimulation of FN.

Late Response of Fyn Activation by FN Requires de Novo Protein Synthesis—To illustrate molecular mechanisms underlying FN-stimulated redistribution of cortactin and formation of lamellipodia or fillopodia in highly metastatic cells X21 and M2, we explored the activity of Fyn under FN stimulation by in vitro kinase assay with poly(Glu-Tyr). In X21, Fyn activity was relatively high when cells were at attachment (Fig. 9A, lane 1), whereas it was sharply reduced when cells were detached and kept in suspension for 30 min (Fig. 9A, lane 2). The activity of Fyn was restored 2 or 5 h after cells were plated on FN (Fig. 9A, lanes 5 and 6). Corresponding to the activation of Fyn, tyrosine phosphorylation of cortactin also exhibited a similar time course (Fig. 9A, lower panel). A similar pattern of activation of Fyn and tyrosine phosphorylation of cortactin by FN was also observed in M2.2 In contrast, levels of Fyn activity were not significantly changed in C10 and C19 after each indicated time plated on FN (Fig. 9B).2 Although tyrosine phosphorylation of cortactin was abolished by detachment, no obvious peaks in tyrosine phosphorylation of cortactin after each indicated time plated on FN were observed in these cells (Fig. 9B, lower panel). Preceding elevated Fyn activity at 2–5 h after stimulation, the expression level of cortactin was also increased from 1 to 2 h in X21 (Fig. 9A, lower panel). The same results were observed in M2.2

The late phase activation of Fyn demonstrated by both M2 and X21 on FN raised the possibility of yet unidentified regulatory mechanisms in integrin-mediated Fyn activation. To determine whether de novo synthesis of regulatory proteins in this process is required, we investigated Fyn activity in the presence of cycloheximide (CHX), a protein synthesis inhibitor. As expected, elevated expression of cortactin induced by integrin stimulation was antagonized by CHX at 5 h after plating (Fig. 9C, lower panel, lane 6). At the same time, it was observed that activation of Fyn was significantly blocked by the treatment of CHX at 5 h after plating (Fig. 9C, upper panel, lane 8) compared with the control (Fig. 9C, upper panel, lane 4). Tyrosine phosphorylation of cortactin was also down-regulated by CHX treatment (Fig. 9C, lower panel, lane 6). The same results were observed in M2.2 These findings indicate that de novo protein synthesis is involved in the late phase activation of Fyn by FN stimulation.

**DISCUSSION**

Previous studies have shown that Fyn is an essential regulator in integrin-mediated processes including actin cytoskeleton organization, cell migration, and adhesion in normal cells (23–25). A recent study also demonstrated that Fyn is prerequisite for normal keratinocyte migration and squamous carcinoma invasion (26). However, little is known regarding the functional roles of Fyn in tumor metastasis. In this study, in contrast to Src, Yes, and Hck, enhanced levels of Fyn activity and late phase activations of Fyn after stimulation by FN were observed in highly metastatic murine melanoma cell lines. Activated Fyn caused hyperphosphorylation of cortactin and formation of stable complex between Fyn and cortactin in these metastatic cell lines, indicating that Fyn and cortactin are key molecules in the integrin signaling pathway during the development of cancer metastasis.

Activation of Fyn by integrin signaling is mediated by many kinds of proteins involved in integrin signaling, which includes caveolin 1, tyrosine kinases, FAK, or receptor protein tyrosine phosphatase α (23, 25, 27). It has been observed in several studies that the activity of Src is enhanced 20–40 min following FN stimulation in NIH 3T3 fibroblasts (28–31). In this study, it was observed that Fyn activation occurs as late as 2–5 h after fibronectin stimulation in metastatic melanoma cell lines, and integrin-mediated activation of Fyn requires de novo protein synthesis. One possibility is that the up-regulation of cortactin that occurred approximately 1–2 h after stimulation is a primary event that triggers the activation of Fyn and phosphorylation of cortactin itself. As a special substrate associated with Fyn in metastatic cells, it is possible that cortactin regulates the activity of Fyn through physical associations to SH2 of Fyn. Another possibility is that a third molecule(s), which is specifically expressed in metastatic cell lines under stimulation of integrin, might regulate activation of Fyn, although this protein(s) has not been identified. In this paper, we do not demonstrate that cortactin is a direct substrate of Fyn kinase. According to a recent paper, tyrosine phosphorylation of cortactin occurs via activation of Rac1 by c-Src in C3H fibroblasts (38). Therefore, there is a possibility that cortactin phosphorylation by Fyn is regulated in a similar indirect manner in highly metastatic melanoma cell lines.

Cortactin was first identified as a p80/85-kDa v-Src substrate in chicken embryo cells transformed by v-Src oncopogene (32). It was implicated in the progression of breast tumors through gene amplification at chromosome 11q13 (33). The importance of tyrosine phosphorylation of cortactin in metastasis was also implicated in a recent report (34). In this study, overexpression and enhanced tyrosine phosphorylation of cortactin in highly metastatic cells was observed, and it was also clarified that cortactin was specifically associated with Fyn in cells with high metastatic potential. When the activity of Fyn was inhibited by PP2, tyrosine phosphorylation of cortactin was also down-regulated. Moreover, corresponding to up-regulated integrin-mediated membrane redistribution of cortactin, co-localized cortactin and Fyn at cell membrane protrusions in highly metastatic cells were also observed. Taken together, these results indicate that cortactin is associated with Fyn in integrin-mediated signaling processes in cancer metastasis. Previous reports have shown that cortactin is primarily localized within peripheral cell structures such as lamellipodia, pseudopodia, and membrane ruffles and has been suggested to operate as a potential regulator in integrin-mediated processes including actin cytoskeleton organization, cell migration, and adhesion in normal cells.
linker between membrane-located receptors and cytoskeleton, primarily affecting cell motility and invasion (11, 12). In this study, corresponding to enhanced cell motility, co-localized cortactin with actin filaments at lamellipodia or filopodia was also observed in highly metastatic cells. Studies in normal cells have shown that cortactin was actively involved in lamellipodia formation (20–22). Lamellipodia and filopodia are membrane structures formed during cell spreading and migration (35). It can be deduced from these reports that cortactin plays important roles in cell migration through its roles in integrin-stimulated actin cytoskeleton rearrangements during cancer metastasis. Support for this deduction is provided by the study of overexpression of cortactin in human tumors or NIH 3T3 fibroblasts, which has been shown to result in increased cell motility (36, 37). Overall, results in this study indicate that the Fyn-cortactin pathway is specifically activated for signaling of cell migration and spreading during the progression of cancer metastasis.

Identification of specific involvements of Fyn and cortactin in integrin signaling pathways in metastatic melanoma cell lines provided important information for the understanding of mechanisms underlying the progression of cancer metastasis. Further studies are necessary to elucidate what is produced de novo for the activation of Fyn and how it affects Fyn activity stimulated by integrin in metastasis. Moreover, to fully clarify their roles in metastasis and to provide further information for cancer therapy, the effect of blocking tyrosine phosphorylation of cortactin or suppressing expression of cortactin on the metastasis potential also needs to be further investigated.

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