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Essential Roles of Lyn in Fibronectin-Mediated Filamentous Actin Assembly and Cell Motility in Mast Cells

Takeshi Suzuki,* Shunsuke Shoji,* Kazuhiko Yamamoto,* Shigeyuki Nada,† Masato Okada,† Tadashi Yamamoto,‡ and Zen-ichiro Honda*‡

Although the requirement for c-Src in extracellular matrix (ECM)-mediated fibroblast motility has been well established, the roles of hemopoietic Src family protein tyrosine kinases in leukocyte migration have not been fully elucidated. To address the issue, we analyzed fibronectin (Fn)-mediated adhesion signaling in rat basophilic leukemia (RBL) 2H3 cells overexpressing 1) Csk, 2) a membrane-anchored, gain-of-function Csk (mCsk), and 3) a kinase-defective mCsk (mCsk(−)). Parent RBL2H3 cells, expressing autoactivated c-kit, readily adhered to Fn-coated surface, developed typical leukocyte adhesion machinery (podosome), and migrated toward Fn without cytokine priming, thus provided a simple experimental system to analyze Fn-mediated outside-in signaling. While overexpression of Csk or the Csk mutants did not significantly affect cell adhesion to the Fn surface or α5 integrin recruitment to the attachment sites, Csk suppressed and mCsk almost abolished Fn-mediated tyrosine phosphorylation of paxillin, filamentous actin assembly to podosomes, and cell migration, but mCsk(−) did not. Coexpression of LynA devoid of C-terminal negative regulatory tyrosine in mCsk cells successfully restored Fn-mediated podosome formation and cell migration. Coexpression of c-Src lacking the C-terminal tyrosine reconstructed podosomes, but could not restore the cell migration regardless of its expression level. Collectively, these observations provide evidence that Src family protein tyrosine kinases are required, and that Lyn can transmit sufficient signal for Fn-mediated cytoskeletal changes leading to cell locomotion in RBL2H3 cells, and they suggest that Lyn and c-Src are differentially involved in cell motility. The Journal of Immunology, 1998, 161: 3694–3701.

Interactions of leukocyte surface integrins with extracellular matrices (ECMs) or with their counter-receptors on endothelial cells are critical steps for inflammatory responses (1–4). The integrin engagement induces leukocyte adhesion and locomotion (5–8), augments the release of specific granules and mediators (5, 9–11), and up-regulates the expression of proinflammatory cytokines (12). A characteristic event following integrin engagement is the assembly of multimolecular adhesion machinery at the cytoplasmic surface of ECM-integrin attachment sites. The most thoroughly characterized example of the adhesion machinery is the focal adhesion (FA) in fibroblasts. FA serves as an attachment site for the long actin filaments and is newly formed in the dynamic cell protrusions (13, 14). FA is composed of integrins, multiple cytoskeletal, and adaptor proteins as well as several signaling molecules, such as protein kinases and small molecular GTPases (14–16). These characteristics have implicated FA as a key signaling complex in structural integrity and also in cell locomotion.

One mechanism underlying the molecular assembly is tyrosine phosphorylation of cytoskeletal and adaptor proteins, which provides sites for the recruitment of Src homology (SH) 2 domains (16, 17). Evidence has been accumulated for the pivotal roles of c-Src in the phosphorylation events in fibroblasts. In the basal state, c-Src activity is suppressed by the phosphorylation of its C-terminal negative regulatory tyrosine (18), and the reaction is catalyzed by C-terminal Src kinase (Csk) (19–21). When cells adhere to fibronectin (Fn)-coated surface, c-Src is activated (22, 23) and is redistributed from cytosol to FAs (24). c-Src and FA kinase colocalized at FA seem to co-ordinately phosphorylate cytoskeletal and adaptor proteins, such as paxillin and p130Cas, thus amplifying the molecular assembly (15, 17, 25–29). In addition, c-Srcmutant fibroblasts exhibit defective spreading and migration on ECMS, indicating the facilitating roles of c-Src in integrin-mediated fibroblast locomotion (22, 23, 30, 31).

Compared with the established roles of c-Src in the adhesion events in fibroblasts, the functional significance of hemopoietic Src family kinases in the signaling has not been fully explored. Recent studies have revealed that Lyn and Fgr are activated and translocated to cytoskeletal fraction (32) and are colocalized with Syk upon neutrophil adhesion over fibrinogen-coated surface (33). By using Fgr and Hck double-knockout mice, Lowell et al. have successfully shown that these hemopoietic Src kinases are involved in neutrophil spreading and O2 release through various integrins, and that their roles are overlapping (34). However, it has not been fully elucidated whether Src family kinases are required for ECM-mediated assembly of leukocyte adhesion machinery (podosome) (4) and cell motility or whether hemopoietic Src family kinase could transmit signals leading to these events. In the current study we have examined these issues through the analysis of rat basophilic...
leukemia (RBL-2H3) mast cell lines overexpressing Csk, a gain-of-function, membrane-anchored Csk mutant (mCsk) (35, 36), and a kinase-defective mCsk (36). We show herein evidence suggesting that Src family kinases are required, and that Lyn could transmit sufficient signal for Fn-mediated podosome formation and cell migration.

Materials and Methods

Materials

All the culture media and Geneticin were purchased from Life Technologies Oriental (Osaka, Japan). FCS was obtained from Equitech (Ingram, TX). Bovine plasma Fn was purchased from Biomedical Technologies (Sturbridge, MA). Type I collagen was obtained from Nitta Gelatin (Osaka, Japan). Fatty acid-free BSA and Latex beads were purchased from Sigma (St. Louis, MO). Rhodamine-phalloidin was obtained from Molecular Probes (Eugene, OR). Protein G-Sepharose was obtained from Pharmacia-LKB (Uppsala, Sweden).

Antibodies

Anti-integrin α5 polyclonal Ab, AB1928P, was obtained from Chemicon (Temecula, CA). Anti-paxillin mAb was purchased from Transduction Laboratories (Lexington, KY). Anti-phosphoepitope mAb, 4G10, was obtained from ICN (Costa Mesa, CA). Anti-c-Myc mAb (9E10) was purchased from Boehringer Mannheim (Indianapolis, IN). FITC-conjugated goat anti-rabbit IgG (heavy + light chains) Ab and FITC-conjugated rabbit anti-mouse IgG (heavy + light chains) Ab were purchased from MBL (Nagoya, Japan). Anti-Csk polyclonal Ab was prepared as previously described (19, 20).

Preparation of expression plasmids and stable expression in RBL2H3 cells

RBL2H3 cells were maintained in DMEM supplemented with 10% FCS, 100 U/ml of penicillin, and 100 µg/ml of streptomycin at 37°C in humidified 5% CO2 as previously described (36). RBL2H3 cells line overexpressing cDNAs for rat Csk, a kinase-defective Csk (Csk(α5–512)), membrane-anchored Csk (Csk(α5–512) in which Lyn(α5–512) is replaced with Arg, a mCsk possessing myristylation signal sequence of rat c-Src (c-Src tag: (M)GSNKSKPKDASQRRR), and its kinase-defective form (mCsk(α5–512)) were described previously (36).

To create mutated rat c-Src and human Lyn whose C-terminal amino acids (amino acids 527–536 for rat c-Src and amino acids 505–512 for human Lyn A) possessing the negative regulatory tyrosine sequence of rat c-Src (c-Src tag: (M)GSNKSKPKDASQRRR), and its kinase-defective form (mCsk(α5–512)) were described previously (36). 36.

Preparation of expression plasmids and stable expression in RBL2H3 cells

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To create mutated rat c-Src and human Lyn A whose C-terminal amino acids (amino acids 527–536 for rat c-Src and amino acids 505–512 for human Lyn A) possessing the negative regulatory tyrosine sequence were replaced with a myc epitope tag (T3SVEDEKLISEEDLN), the SpaI site was artificially introduced at the junctional sites of the cDNAs using QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s protocol. The C-terminal short nucleotide sequences were cut out with SpaI and oligonucleotides coding for the myc epitope tag flanked with SpaI and XbaI were ligated with the cDNAs at the C-termini. The resultant cDNAs, termed ΔSsrc and ΔLyn, were subcloned into pBluescript SK+(Stratagene). Clones without misincorporation of the nucleotides were selected, subcloned into an expression vector, pCAGGS (provided by Dr. Miyazaki) (37), and stably introduced into mCsk-expressing cells by cotransfection with a puromycin-resistant vector. Puromycin-resistant clones were screened by immunoblotting with anti-myc mAb (9E10) (38). Multiple independent cell lines expressing ΔSsrc or ΔLyn were established and used for functional studies.

Cell adhesion experiments

Twenty-four- or 96-well flat-bottom polystyrene plates without chemical or mechanical coating (Iwaki, Chiba, Japan) were coated with various concentrations of Fn in PBS overnight at 4°C. Wells were washed once with PBS, incubated with 20 mg/ml BSA in PBS for 1 h at 37°C for blocking, and again washed twice with PBS and once with DMEM before experiments. Adherent RBL2H3 cells serum starved for 24 h were washed twice with PBS and detached from dishes by treatment with HBSS containing 0.05% trypsin and 0.53 mM EDTA (Life Technologies Oriental) at 37°C for 5 min. Cells were harvested with gentle pipetting with DMEM, and trypsin was immediately neutralized with 0.5 mg/ml soybean trypsin inhibitor. Cells were washed once with DMEM and kept at suspension in 0.05% trypsin and 0.53 mM EDTA (Life Technologies Oriental) at 37°C. For immunostaining, cells were incubated with 20 mg/ml BSA in PBS for 30 min at 37°C for blocking and incubated with first Abs in the blocking solution for 1 h. After washing with an excess volume of 0.1% Triton X-100 in PBS, the first Abs were probed with FITC-conjugated second Abs. The samples were mounted, viewed, and photographed with an Olympus BX50 microscope (Olympus, New Hyde Park, NY) equipped with epifluorescent filters.

Loading of cells with Fn-coated microbeads

Cell loading with Fn-coated microbeads was conducted essentially as previously described (40). Small (mean diameter, 3 µm) or large (mean diameter, 11.9 µm) latex beads were coated with 50 µg/ml of Fn before the assay (40). Cells were cultured on type I collagen-coated Lab-Tek chamber slides (Nunc, Naperville, IL) overnight in serum starved for 3 h. The adherent cells were washed twice with PBS and fixed with PBS containing 4% formaldehyde and 5% sucrose for 20 min at 25°C. The fixed cells were rinsed twice with PBS, permeabilized with 0.1% Triton X-100 in PBS for 5 min at 25°C, and again rinsed twice with PBS. Filamentous actin (F-actin) was stained with rhodamine-conjugated phalloidin for 30 min at 37°C. For immunostaining, cells were incubated with 20 mg/ml BSA in PBS for 30 min at 37°C for blocking and incubated with first Abs in the blocking solution for 1 h. After washing with an excess volume of 0.1% Triton X-100 in PBS, the first Abs were probed with FITC-conjugated second Abs. The samples were mounted, viewed, and photographed with an Olympus BX50 microscope (Olympus, New Hyde Park, NY) equipped with epifluorescent filters.

Cell migration assay

Cell migration assay using a chemotaxis micro chamber (41) was performed as described previously (42). The lower wells of a 48-well chemotaxis chamber (Neuroprobe, Pleasanton, CA) were filled with 100 µg/ml of Fn in DMEM or with DMEM alone (control), and a polycarbonate filter (5 µm pore size; Neuroprobe) was layered onto the wells. After assembly of the upper chamber, 50 µl of cell suspension (1 × 105/ml in DMEM) was added to the upper wells. The chambers were incubated for the indicated times at 37°C in humidified 5% CO2. After the incubations, the filter was removed, and cells that remained at the upper surface of the filter were scraped out. The filter was fixed and stained with Diff-Quik (International Reagents, Kobe, Japan), and cells that migrated through pores to the lower side of the filter were counted by light microscopy.
Clones (p62 are expressed as the mean mCsk, and mCsk(2) effectant; lane 4, mCsk transfectant; lane 4, mCsk(-- transfectant). The migrated positions of Csk, mCsk, and mCsk(-- are indicated by arrows.

FIGURE 1. Expression of Csk and its mutants in RBL2H3 cells. Cells were lysed with Nonidet P-40 lysis buffer, standardized for protein concentration, and subjected to immunoblotting with polyclonal anti-Csk Ab (lane 1, wild-type RBL2H3; lane 2, Csk transfectant; lane 3, mCsk transfectant; lane 4, mCsk(-- transfectant). The migrated positions of Csk, mCsk, and mCsk(-- are indicated by arrows.

Results

Adhesion of RBL2H3 cells to Fn-coated surface is not affected by the expression of Csk, mCsk, or mCsk(--)

In preliminary experiments we have confirmed that RBL2H3 cells, expressing constitutively active c-kits, readily attach to and migrate on Fn-coated surface without cytokine priming (43–45). These characteristics provided a simple experimental system to explore the Fn-induced outside-in signal. To analyze the roles of Src-family kinases in the signaling cascade, we used RBL2H3 cell lines overexpressing Csk, a mCsk (36, 46), and a kinase-defective mCsk functions as a dominant negative molecule (36). A representative immunoblotting of RBL2H3 cells expressing these molecules is shown in Figure 1.

We first tested whether Csk and its mutants affect cell adhesion. As shown in Figure 2, expression of Csk, mCsk, or mCsk(-- did not alter cell adhesion to the dishes coated with various concentrations of Fn (0.46–10 μg/ml) at 60 min or cell adhesion to 10 μg/ml Fn-coated dishes during incubation periods of 10–80 min.

These findings were reproducible in at least two clones for each Csk-based molecule. It was also noted that recruitment of α5 integrin subunit, a component of mast cell Fn receptor (very late Ag-5), to Fn attachment sites was not inhibited by Csk or its mutants (see Fig. 4, B and C). These observations indicate that the changes in Csk activity did not alter RBL2H3 cell adhesion to Fn-coated surface.

Csk inhibits adhesion-dependent protein tyrosine phosphorylation

We next explored whether Csk and its mutants affect Fn-mediated protein tyrosine phosphorylation. As shown in Figure 3A, adhesion of parent RBL2H3 cells to Fn-coated surface induced tyrosine phosphorylation of about 70 kDa (indicated by double asterisks in Figure 3) and about 120 kDa (indicated by single asterisk) proteins, findings consistent with earlier reports (9, 49). In Csk transfectants, these signals were reduced and delayed, and these inhibitory effects were more prominent in mCsk transfectants. Expression of mCsk(--) did not appreciably suppress the approximately 120-kDa signal; rather, it augmented the approximately 70-kDa signal. In addition, a constitutive tyrosine phosphorylation of the approximately 60-kDa protein(s) (indicated by # in Figure 3) was observed in mCsk(--) cells. This protein(s) was not identified in the current study, but it might correspond to a v-Src substrate, p62 dok (50, 51).

An adaptor protein, paxillin, has been assumed to be a c-Src substrate (28, 29) and has been presumed to play a crucial role in FA assembly (52). As shown in Figure 3B, tyrosine phosphorylation of paxillin was inhibited by Csk and was more profoundly inhibited by mCsk. The mCsk(--) did not exert a negative effect; instead, it augmented the basal phosphorylation level. These findings were reproducible at least two clones for each Csk-based molecule.

Csk inhibits podosome formation and cell locomotion stimulated by Fn

We next explored whether the altered Csk activity affects Fn-mediated formation of adhesion machinery and cell locomotion. As shown in Figure 4Aa, RBL2H3 cells developed characteristic dot-like F-actin assembly (podosome) at Fn attachment sites. Staining with anti-vinculin Ab revealed colocalization of the F-actin bundles with vinculin (not shown). In Csk- and mCsk-expressing cells,
cells were fully spread (Fig. 4, A). F-actin assembly to podosomes was markedly reduced, even when
mCsk cells were stimulated as described above. Adherent cells were lysed with 1% Nonidet P-40 lysis buffer, stan-
dardized for protein concentration, and immunoprecipitated with 4G10 as described in Materials and Methods.

Differential effects of Lyn and c-Src devoid of C-terminal negative regulatory tyrosine on mCsk-suppressed podosome formation and cell migration

The stepwise inhibitory effects of Csk and mCsk on Fn-mediated podosome formation and cell migration strongly suggested that activation (or structural change to an ‘open’ conformation (22, 23)) of Src family protein tyrosine kinases is required for these functions. To further evaluate the hypothesis, we tested whether Src family kinases lacking C-terminal negative regulatory tyrosine (a substrate for Csk (19)) could restore the impaired functions in mCsk cells. To this end, we created mutated Lyn and c-Src, two major Src family kinases expressed in RBL2H3 cells (55) whose corresponding C-terminal amino acid sequences (amino acids 505–512 for LynA and amino acids 527–536 for c-Src) were re-
placed with a c-src epitope tag sequence (Fig. 6Aii). These muta-
tants (termed ΔLyn and ΔSrc, respectively) or a puromycin-resis-
tant vector alone were stably introduced into the mCsk-expressing cells, and clones with high (hi.) and low (lo.) expression levels of ΔLyn and ΔSrc were obtained. A representative immunoblotting with the anti-c-src tag Ab of vector controls and cells expressing ΔSrc or ΔLyn is shown in Figure 6Aii.

When these cells were loaded with Fn-coated beads, tyrosine-
phosphorylated proteins (PY) and dot-like F-actin bundles were clearly observed at the attachment sites in ΔSrc- or ΔLyn-expressing mCsk cells, but not in vector controls (Fig. 6B). The number of positively stained beads was scored, and the data are summarized in Figure 6C. ΔLyn restored the impaired recruitment of tyrosine-
phosphorylated proteins and F-actin by mCsk, whereas ΔSrc aug-
mented it.

We next tested whether ΔSrc or ΔLyn could rescue the defective cell migration toward Fn. As shown in Figure 6D, Fn-mediated migration in the vector control cells was kept at low levels, as expected. Coexpression of ΔLyn restored the impaired migration of mCsk cells, or, rather, enhanced it, whereas ΔSrc expression did not regardless of their expression levels. Apparently, ΔSrc expres-
sion further inhibited migration to lower levels than those in vector controls.

Discussion

As an approach to study the roles of Src family kinases in integrin-
induced leukocyte motility, Fn-mediated outside-in signaling was investigated in RBL2H3 cells overexpressing Csk, mCsk, or mCsk(−). In the previous study, it was confirmed that basal and high affinity IgE receptor-stimulated Lyn activities are stepwise suppressed in Csk- and mCsk-overexpressing cells (36). RBL2H3 cells readily adhere to Fn-coated surface, develop F-actin assembly to podosomes (see Fig. 4A), and migrate toward Fn without cyto-
kine priming (inside-out signaling). These characteristics of RBL2H3 cells have provided a suitable experimental system to focus upon outside-in signaling. Our data indicate that overexpression of Csk or the mutated Cskds do not significantly affect the interaction of Fn with α5 integrin in RBL2H3 cells. Firstly, Csk and mCsk did not inhibit the recruitment of α5 integrin to the attachment sites with Fn-coated beads. Similar observations have been reported in human foreskin fibroblasts, where treatment of the cells with herbimycin A, a relatively Src-selective tyrosine kinase
FIGURE 4. Immunofluorescence studies of molecular assembly to Fn adhesion sites. A. Cells were cultured on Fn (30 μg/ml)-coated dishes for 24 h, and F-actin accumulated on the ventral surfaces was stained with rhodamine-conjugated phalloidin. a, Wild-type cells; b, Csk transfectant; c, mCsk transfectant; d, mCsk(−) transfectant. Scale bar = 10 μm. B. Cells were plated onto type I collagen (a, e, i, and m, wild-type cells; b, f, j, and n, Csk transfectant; c, g, k, and o, mCsk transfectant; d, h, l, and p, mCsk(−) transfectant) and incubated with small (mean diameter, 3 μm; a–l) or large (mean diameter, 11.9 μm; m–p) beads coated with 50 μg/ml Fn for 30 min. Localization of integrin α5 (a–d) and tyrosine-phosphorylated proteins (e–h) was detected by indirect immunofluorescent staining. Localization of F-actin was detected by rhodamine-labeled phalloidin (i–l and m–p). In the experiments using small beads (a–l), views were focused on the equator of the bead marked by a white arrowhead. Highlighted black arrowheads indicate other beads bound to the cells. Magnified views were shown in the insets. In the experiments in which large beads were used (m–p), views were focused around the basal attachment sites of the beads. Equators of the beads were drawn as a white circle. Scale bar = 10 μm. C. Beads positively stained for integrin α5, tyrosine-phosphorylated proteins, and F-actin were scored. The ordinate indicates the percentage of beads positive for immunofluorescence for each molecule. At least 50 beads were scored in each experiment, and data are expressed as the mean ± SD from three separate experiments. *, p < 0.01 compared with parent RBL2H3 cells (RBL-WT) by t test.
inhibitor, does not suppress α5 integrin assembly to the Fn-coated bead surface (40). Secondly, cell adhesion to the Fn-coated dish was not significantly affected by the overexpression of the Csk-based molecules. It was also noted that cell spreading over Fn or type I collagen was not appreciably influenced by Csk or by mCsk (Fig. 4, A and B, and data not shown). As for the roles of Src family kinases in the adhesion properties of hemopoietic cells, Lowell et al. have demonstrated that Hck<sup>mol</sup>Fgr<sup>mol</sup> murine neutrophils stimulated with TNF are defective in β<sub>2</sub> integrin-mediated tight adhesion and cell spreading, while these functions are preserved when the cells are primed with phorbol ester (34, 56). These findings suggest that requirement of Src family kinases for these functions are dependent on the signaling pathways through which integrins are activated, and that Src family kinases are not critically involved in protein kinase C-stimulated cell adhesion on ECMs. Although the mechanisms of the integrin activation in RBL2H3 cells should be further clarified, autoactivation of c-Kit in the cells (43), that potentially leads to the protein kinase C pathway via phospholipase Cγ and phosphatidyl inositol 3-kinase (4, 44, 45) is a feasible candidate that could explain the constitutive adhesion of RBL2H3 cells and also the lack of inhibitory effects of Csk on cell adhesion.

Although the initial interaction of α<sub>5</sub> integrin with Fn was not affected by Csk or mCsk overexpression, postintegrin signaling was significantly inhibited by the augmented Csk activity. Firstly, it was found that Fn-mediated tyrosine phosphorylation of cellular proteins, including paxillin, was stepwise inhibited by Csk and mCsk, but not by mCsk(−). Secondly, the assay using Fn-coated microbeads quantitatively showed that Csk and mCsk strongly suppressed the recruitment of tyrosine-phosphorylated proteins and short F-actin bundles to podosomes, but mCsk(−) did not. In addition, the assembly of the short F-actin bundle was hardly detectable at the ventral surface of Csk- and mCsk-expressing cells even under fully spread conditions (Fig. 4A), thereby indicating that cell spreading and podosome formation could be separable events. Podosome was originally described in v-Src-transformed tumor cells (57) and has been assumed to be a key signaling machinery to facilitate the motility of hemopoietic cells (4). The Csk-mediated inhibition of podosome formation might suggest that development of podosomes in hemopoietic cells is also regulated by the elevated basal activity of Src family kinases in these cell types (36, 58). Thirdly, we showed that Csk inhibited and mCsk almost abolished Fn-dependent cell migration, findings consistent with the essential roles of podosomes for cell migration. The mCsk(−) only partially reduced the migration. Collectively, Csk and mCsk inhibited the series of events in a stepwise manner that was dependent on the catalytic activity. Although the substrate for Csk is not strictly confined to Src family kinases (59), the correlation between the Csk activity and the extent of inhibition strongly suggest that Src family kinases are required for the signaling cascade. One exception in the correlation was that the cell locomotion was slightly suppressed by mCsk(−), albeit the inhibition was reproducibly smaller than that by Csk or mCsk. The reason for the apparent discrepancy is not clear, but a possible explanation might be that the augmented intrinsic c-Src activity by the dominant negative effects of mCsk(−) (36) hampered the cell migration (see below).

Through the analysis of mCsk-expressing RBL2H3 cells, it was found that these cells provided a condition where the initial interaction of Fn with α<sub>5</sub> integrin was preserved, whereas Fn-mediated podosome formation and cell locomotion were almost abolished. As an approach to further evaluate the roles of Lyn and c-Src, two major Src family kinases expressed in RBL2H3 cells (55), we introduced epitope-tagged, constitutively activated forms of Lyn and c-Src (ΔLyn and ΔSrc) into mCsk-expressing cells. It has been suggested that the augmented basal catalytic activity of such C-terminal tyrosine-unsphosphorylatable kinase is not sufficient to initiate organized cellular functions, including B cell receptor-mediated calcium mobilization (58) or TCR-mediated IL-2 production (46), and that participation of these molecules in receptor-originated signaling machinery is still required to transmit these remote signals. Therefore, this approach could provide information about whether once activated kinases could be involved in a given signaling pathway. We found that ΔLyn successfully restored mCsk-suppressed F-actin assembly to podosomes and also cell migration toward Fn. These findings strongly suggest that Lyn activation could transmit sufficient signal to Fn-mediated cytoskeletal changes leading to cell migration in RBL2H3 cells. On the contrary, ΔSrc restored the F-actin assembly, but could not rescue the impaired cell migration regardless of its expression levels. Apparently, ΔSrc further suppressed it. Therefore, continuous activation of c-Src seems to be sufficient for F-actin aggregation, but inhibitory to cell motility. Obviously, the constitutive activation of the mutated c-Src could not fully reproduce the highly regulated innate signaling pathway. Thus, these negative results do not mean that receptor-activated c-Src transmits minimal or negative signal to the cell migration. However, the opposite effects of the two autoactive kinases still raise the possibility that Lyn and c-Src differentially participate in cell motility. Considering the overlapping roles of ΔSrc and ΔLyn in the podosome assembly, continuous activation of Lyn and c-Src might oppositely influence the turnover of the adhesion machinery (13, 54). It should be noted that mCsk/ΔLyn lo. cells migrated significantly more than mCsk/ΔLyn hi. cells (Fig. 6D). The inverse correlation between the expression levels of ΔLyn and the migration levels suggests that excessive activation of Lyn is inhibitory to cell migration. Although Lyn activity is required for podosome formation and cell migration, excessive activation of Lyn might stabilize the adhesion machinery that leads to decreased cell mobility. Considering that ΔSrc restored podosome formation more markedly than ΔLyn did (Fig. 6C), and that ΔSrc inhibited cell motility (Fig. 6D), it might be argued that activation of c-Src more potently fixes the adhesion machinery than Lyn does. Further study is required to understand the precise mode of actions of Lyn and c-Src in the adhesion signaling and to elucidate the structural determinants responsible for the differential effects of these kinases.
In summary, this study has provided evidence that Src family kinases are required, and activation of Lyn could transmit signal for Fn-mediated cytoskeletal changes leading to RBL2H3 cell motility. Considering that hemopoietic cells express multiple Src family kinases in a lineage-specific manner (60), these family members might possess different functions in ECM-mediated signaling cascade that leads to cell migration. In addition, in view of the highly redundant roles of Src family kinases in a number of biological functions (27, 34, 61), the experimental design in which mCsk-expressing cells are used as a functionally Src family kinase-deficient background could complement the knowledge obtained from gene knockout studies.

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References


