Src Kinase Determines the Dynamic Exchange of the Docking Protein NEDD9 (Neural Precursor Cell Expressed Developmentally Down-regulated Gene 9) at Focal Adhesions*

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**Background:** Dynamic exchange provides a mechanism for rapidly reorganizing macromolecular structures.

**Results:** Exchange of the focal adhesion-targeted protein NEDD9 between the cytoplasm and focal adhesions is faster in the absence of Src kinase activity.

**Conclusion:** Src kinase modulates the transit time of NEDD9 at focal adhesion sites.

**Significance:** This is a new function for Src kinase in cell migration.

Dynamic exchange of molecules between the cytoplasm and integrin-based focal adhesions provides a rapid response system for modulating cell adhesion. Increased residency time of molecules that regulate adhesion turnover contributes to adhesion stability, ultimately determining migration speed across two-dimensional surfaces. In the present study we test the role of Src kinase in regulating dynamic exchange of the focal adhesion protein NEDD9/HEF1/Cas-L. Using either chemical inhibition or fibroblasts genetically null for Src together with fluorescence recovery after photobleaching (FRAP), we find that Src significantly reduces NEDD9 exchange at focal adhesions. Analysis of NEDD9 mutant constructs with the two major Src-interacting domains disabled revealed the greatest effects were due to the NEDD9 SH2 binding domain. This correlated with a significant change in two-dimensional migratory speed. Given the emerging role of NEDD9 as a regulator of focal adhesion stability, the time of NEDD9 association at the focal adhesions is key in modulating rates of migration and invasion. Our study suggests that Src kinase activity determines NEDD9 exchange at focal adhesions and may similarly modulate other focal adhesion-targeted Src substrates to regulate cell migration.

Cell adhesion to the extracellular matrix is an essential component of cell migration and is chiefly determined by integrin receptor binding to extracellular matrix ligands. Ligand-bound and cross-linked integrins together with the associated signaling molecules and polymerized actin filaments in the cell interior comprise specialized adhesion sites known as focal adhesions. These sites both tether the cells to the underlying matrix and transmit signals bi-directionally between the matrix and the cell cytoplasm (1). During cell motility, focal adhesions must form and turnover, allowing grip and release of the underlying matrix and translocation of the cell bodies. Rapid exchange of proteins between focal adhesions and the cytoplasm (measured in time scales of s) facilitates the reorganization of the macromolecular focal adhesions in response to conditions that favor migration (2, 3). The time with which focal adhesion-stabilizing molecules reside in the focal adhesion (residency time), therefore, represents an important mechanism to modulate adhesion to the extracellular matrix and consequently migratory behavior.

The balance of focal adhesion assembly *versus* turnover determines migration speed across two-dimensional planar surfaces (4–7). This is a biphasic relationship where either too little or too much adhesion slows two-dimensional cell speed, and maximal migration speeds are observed at intermediate attachment strengths (8, 9). Thus conditions that alter the turnover rate of focal adhesions can correspondingly tune cell migration speed. Investigations of a number of focal adhesion molecules have confirmed that many serve to regulate focal adhesion turnover rates (10). Whereas a number of molecules stimulate focal adhesion turnover, our recent data have revealed that the focal adhesion-associated molecule NEDD94/HEF1/Cas-L instead stabilizes focal adhesions and can thus tune two-dimensional migration speed (11, 12).

NEDD9 is a docking protein at focal adhesions that is subject to extensive phosphorylation modification by Src kinase (13). NEDD9 encompasses multiple protein-protein interaction domains that facilitate the docking of interacting partner proteins, stimulating a variety of cellular processes, including a

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4 The abbreviations used are: NEDD9, neural precursor cell expressed developmentally down-regulated gene 9; FAK, focal adhesion kinase; FRAP, fluorescence recovery after photo-bleaching; SH2, Src-homology 2 domain; SH2BD, SH2 binding domain; SBD, Src binding domain; MEF, mouse embryo fibroblast.
prominent pro-migratory/pro-metastatic role (13, 14). Recruitment to focal adhesions is achieved through interaction with focal adhesion kinase (FAK) resident at the focal adhesions (15). FAK then phosphorylates a tyrosine motif (DYD) in the NEDD9 C-terminal domain that creates a binding site for Src (the Src binding domain (SBD)) (13). Once docked, Src phosphorylates up to 13 consensus tyrosine phosphorylation sites in the NEDD9 substrate binding domain (SH2BD). This creates docking sites for SH2 domain-containing partner proteins and is an important determinant of NEDD9 regulated cell migration (12, 16–18).

In addition to the well-established role for Src phosphorylation in stimulating NEDD9-mediated signaling cascades, we hypothesized that NEDD9 phosphorylation by Src may also regulate the residency time at focal adhesions. Increased opportunities for interactions between NEDD9 and other focal adhesion molecules as a result of extensive phosphorylation by Src could increase the time in which NEDD9 is tethered at the focal adhesions by stabilizing interactions with partner molecules. Although there have been a handful of studies investigating the exchange rates of focal adhesion molecules, at present there is little understanding of how this is controlled. We have employed Src/Yes/Fyn (Src)-/- mouse embryo fibroblasts (MEFs) and NEDD9-/- MEFs to investigate the role of Src kinase in NEDD9 dynamic exchange at focal adhesions. Our data indicate that Src regulates the transit time of NEDD9 at focal adhesions.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture—Src/Yes/Fyn (SYF-/-) null and FAK-/- MEFs were from American Type Culture Collection (ATCC), and NEDD9 (NEDD9-/-) MEFs have been described (11). All lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with either 10% fetal bovine serum (FBS) (Invitrogen), 1% penicillin/streptomycin (Invitrogen), and 2 µg/ml Fungizone (Invitrogen) or 15% FBS and 1% antibiotic/antimycotic (Invitrogen), at 37 °C and 5% CO₂.

Antibodies—Monoclonal anti-NEDD9 and polyclonal anti-FAK (Cell Signaling Technology), monoclonal anti-paxillin and p130Cas (BD Transduction Laboratories), anti-Src (Clone GD11) (Millipore), monoclonal anti-GFP (Roche Applied Science), monoclonal anti-GFP and polyclonal anti-paxillin Tyr(P)-118 (Invitrogen), anti-β-actin and anti-HSP-70 (Sigma), horseradish peroxidase-conjugated goat secondary antibodies for immunoblot analysis (Amersham Biosciences), and Cy3-conjugated donkey anti-rabbit and Cy5-conjugated donkey anti-mouse IgG secondary antibodies for immunofluorescence (Jackson ImmunoResearch) were used.

Plasmids and Protein Expression—NEDD9 fused to GFP has been previously described (19). The QuikChange kit (Stratagene) was used to mutate NEDD9 Tyr-629 and Tyr-631 to phenylalanines (NEDD9.DFDF). Primer pairs: forward (5’-GCTGGATGGATGACTT-3’) and reverse (5’-TCGAAGTCATCCACCTACCGTCCACCTAGGG-3’) and forward (5’-CGATGGAAGTGGGACCGAAA-3’) and reverse (5’-CGATGGAAGTGGGACCGAAA-3’). SH2BD domain deletion was achieved by synthesizing the sequence encoding the first 67 amino acids (M1-M67) flanked by EcoR1 and Aval restriction sites then joined by restriction enzyme-mediated ligation with the NEDD9 beginning at Ser-358 (deleting the intervening 290 residues). The mutant sequence was subcloned into the same GFP vector backbone as the wild-type sequence. All constructs were confirmed by DNA sequencing. Transient MEF cell transfection was achieved using a nucleofector (Amaxa) and MEF1 nucleofector kit (Lonza).

Immunoprecipitation, Immunoblot, and Immunofluorescence—

Immunoprecipitation was performed using 1 mg of protein lysate extracted in Nonidet P-40 lysis buffer (50 mM Tris (pH 7.5) 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40) freshly supplemented with protease inhibitors (1 mM NA₃VO₄, 1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin). Protein G-agarose was swollen in 10 mM HEPES (pH 7.4) on ice for 30 min, then washed in PBS and resuspended at a concentration of 4 mg/ml in Nonidet P-40 lysis buffer supplemented with protease inhibitors. Cell lysates were precleared with 4 mg of agarose and mixed end-over-end at 4 °C for 1 h. Precleared lysates were incubated with anti-GFP (Roche Applied Science) or mouse IgG for 2 h at 4 °C to which 4 mg of protein G-agarose was added and incubated for a further 16 h. Immunoprecipitates were washed three times with Nonidet P-40 lysis buffer (supplemented with inhibitors) and eluted in SDS-PAGE sample buffer. For all other immunoblotting, PTV lysis buffer protein extraction and immunoblotting were performed as previously described (20). For immunofluorescence, cells cultured overnight on glass coverslips were fixed in 4% paraformaldehyde and permeabilized in PBS, 0.5% bovine serum albumin and 0.2% Triton X. Coverslips were mounted with Fluorsave (Merck-Millipore). Confocal microscopy was performed using a Leica SP5 confocal, 63× oil objective. Images were captured using the Leica LAS-AF software. Cells were imaged at a single optical plane corresponding to focal adhesion positions on the ventral surface.

Fluorescence Recovery after Photobleaching (FRAP)—Transfected cells were cultured overnight at 37 °C with 5% CO₂ in 35-mm glass-bottom dishes (MatTek, Boston, MA). Media were then exchanged for CO₂-independent media supplemented with 15% FBS and antibiotic/antimycotic, and cultures were equilibrated at 37 °C for 1 h. FRAP analysis was performed using either Leica SP5 confocal and 63× oil objective (NEDD9 mutant constructs) or Olympus FV1000 confocal and 60× oil objective (SYF-/- and PP2 experiments), both equipped with a 37 °C environmental chamber. Focal adhesions were photobleached for 3 s with an argon laser at 50%. Images of the bleached area were then recorded every 1.4 s over a minimum 60-s period. Recovery rates were calculated and normalized to single control measurements taken per focal adhesion using either the Leica-supported software LAS-AF or the Olympus Fluoview software. FRAP analysis was performed on cells from three independent experiments on separate days and then pooled to obtain sufficient numbers of focal adhesions for analysis. Src inhibition with PP2 (Sigma) was achieved by incubation with 2 µM PP2 overnight before analysis. Statistical analysis was performed using GraphPad Prism.

Live Cell Imaging, Migration, and Spreading Analysis—

Time-lapse images of transfected cells at low confluence in a 35-mm plastic dish were captured using an ORCA ERG cooled
CCD camera (Hamamatsu SDR Clinical Technology, NSW, Australia) and an Olympus IX81 inverted microscope equipped with a 37 °C environmental chamber. Transmitted light images of transfected cells (identified by initial single fluorescence image) were captured every 5 min over 6 h. Cells undergoing division or apoptosis were excluded from analysis. Nuclear translocation was tracked in the time-lapse image stacks using Metamorph V6.3 software (Molecular Devices).

RESULTS

Src Regulates NEDD9 Exchange at Focal Adhesions—We first confirmed that NEDD9 localization to focal adhesions does not require Src kinase activity by transfecting Src−/− MEFS with GFP-tagged NEDD9. NEDD9 robustly localized to focal adhesions in a similar pattern to that observed in NEDD9+/− MEFS, with strong concordance between the focal adhesion marker paxillin and GFP.NEDD9 subcellular distribution (Fig. 1A). NEDD9 is detected as two phospho forms by Western blot (21, 22), and exogenous GFP.NEDD9 similarly produces a doublet in NEDD9+/− and Src−/− MEFS (Fig. 1A). The small decrease in the upper form of NEDD9 in the Src−/− MEFS was inconsistent between experiments (data not shown).

We next compared GFP-tagged NEDD9 exchange at focal adhesions between Src−/− and NEDD9−/− MEFS by FRAP. For uniformity, all analyses were focused on focal adhesions in regions of protruding cell membrane. Notably, the recovery of GFP.NEDD9 appeared faster in the Src−/− MEFS (Fig. 1, B and C). Quantification of recovery rates confirmed that they were indeed significantly faster (Fig. 1D). To discount the possibility that the differences seen might be due to different cell backgrounds (Src−/− versus NEDD9−/− MEFS) GFP.NEDD9 recovery was next measured in NEDD9−/− MEFS treated with PP2, a chemical inhibitor of Src kinase. Importantly, NEDD9 fluorescence recovery in the presence of PP2 was significantly increased when compared with untreated NEDD9−/− MEFS (Fig. 1, C and D). Because the Src−/− MEFS express endogenous NEDD9, to rule out that the difference reflects changes in the
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FIGURE 2. FRAP analysis of NEDD9ΔCT. A, schematic of NEDD9 WT sequence and C-terminal domain deletion mutant (NEDD9ΔCT). SRR, serine-rich region; CT, C terminus. B, as NEDD9ΔCT does not localize to focal adhesions, cells were co-transfected with mCherry-tagged paxillin to detect focal adhesions. This was used to mask the relevant areas in the GFP images to analyze NEDD9ΔCT dynamics. Shown is an example of mCherry paxillin used to identify relevant areas for photo-bleaching the GFP signal. Scale bars = 5 µm. C, fluorescence recovery of GFP:NEDD9 (black squares) versus GFP:NEDD9ΔCT (white squares) in NEDD9−/− MEFs. D, fluorescence recovery rates for the indicated constructs. Data represent the mean of >43 focal adhesions per construct, and error bars show S.E. ***, p < 0.001, Student’s t test.

total amount of NEDD9 protein, we next compared the dynamics of GFP:NEDD9 in NEDD9−/− MEFs versus matched wild-type MEFs. This revealed identical recovery kinetics between the NEDD9−/− MEFs and the wild-type MEFs (Fig. 1E). Together these data suggest an important role for Src in determining NEDD9 exchange at focal adhesions.

For comparison, we examined GFP:NEDD9 with the C-terminal domain deleted (NEDD9ΔCT). This domain encompasses the focal adhesion targeting domain (23) and is required for focal adhesion localization (24). Cells were co-transfected with mCherry-tagged paxillin to identify the focal adhesion region of interest for photobleaching (Fig. 2, A and B). As anticipated, the NEDD9ΔCT construct exhibited faster exchange in the region of the focal adhesions (Fig. 2, C and D). However, this did not simply reflect free diffusion, as the same analysis of unfused GFP at focal adhesions marked by mCherry paxillin showed an ~10-fold higher exchange rate (GFP,NEDD9ΔCT K = 0.35 ± 0.018 versus GFP K = 2.98 ± 0.24; ***, p < 0.001, Student’s t test). Consequently, despite the lack of the focal adhesion targeting domain (thereby abrogating GFP-positive adhesions), the molecular dynamics were slowed around the focal adhesion relative to unfused GFP. Together these data suggest that localization (concentrated appearance at a focal adhesion) is separable from the rate of exchange between the focal adhesion juxtamembrane region and the cytoplasm.

The NEDD9 SH2 Binding Domain Dominantly Regulates Exchange—Src stimulates focal adhesion turnover (10) and regulates actin filament dynamics (25), in turn controlling actomyosin force that determines adhesion molecule exchange (3). Potentially, therefore, faster NEDD9 exchange in the absence of Src activity may have been secondary to altered actin filament dynamics or Src-dependent focal adhesion turnover. To rule this out we quantified the role of the two direct NEDD9 Src target sites: the SBD (DYDY) and the SH2BD (26). First, the SBD tyrosines were mutated to phenylalanine to prevent phosphorylation (DFDF) (Fig. 3A). Note that NEDD9 does not contain the additional Src SH3 binding that forms the Src bipartite binding domain in other Cas family proteins (13) and so only binds Src via the tyrosine motif. NEDD9.DFDF generates the same doublet pattern on Western blot as seen for the wild-type sequence (Fig. 3A). Immunofluorescence confirmed co-localization of the mutant protein with paxillin-positive focal adhesions at the cell periphery and ventral surface (Fig. 3B). FRAP analysis of GFP:NEDD9.DFDF positive focal adhesions revealed a significantly increased fluorescence recovery rate compared with wild-type NEDD9 (Fig. 4, A–C). Thus phosphorylation of the DYDY motif and subsequent Src kinase docking may slow the rate of NEDD9 molecular exchange at focal adhesions.

Next, we analyzed a NEDD9 SH2BD-deleted mutant (NEDD9ΔSH2BD). As this also deletes the NEDD9 antibody binding epitope, anti-p130Cas antibodies, which bind a conserved epitope (19), were used to confirm expression of the truncated protein (Fig. 3A). Importantly, loss of the substrate binding domain does not affect NEDD9 targeting to the focal adhesions (Fig. 3B). GFP-tagged NEDD9ΔSH2BD co-localized at all regions of paxillin-positive focal adhesions (Fig. 3B). FRAP analysis of this construct revealed a striking increase in the rate of fluorescence recovery (Fig. 4, A–C). Notably, this was also significantly faster than the recovery rate of NEDD9.DFDF (Fig. 4C). We then questioned whether the NEDD9 mutant proteins exhibited an altered mobile fraction, defined as the maximum plateau of fluorescence recovery. Although analysis revealed a significant difference between the maximum plateaus of the NEDD9ΔSH2BD versus NEDD9.DFDF and NEDD9 (Fig. 4D),...
the magnitude of the difference was very small (80 and 82% versus 80 and 79%). Thus in each case ≈20% of the total pool remains immobile, agreeing with earlier descriptions of the stable focal adhesion fraction over a similar time course (27). Notably, the difference between GFP.NEDD9 in the NEDD9/+/MEFs versus the Src+/+/MEFs was considerably larger (Fig. 1C). The relatively smaller difference between the mutant forms and the PP2-treated cells with wild-type NEDD9 suggests differences observed in the Src−/− MEFs may be cell type-specific and suggest that the most important effects of Src may be on exchange. Our data suggest a hierarchy of Src kinase effects; the substrate binding domain has the greatest effects on NEDD9 dynamics at focal adhesions, whereas the Src SH2 binding motif has a more limited role.

Interaction with FAK Regulates NEDD9 Focal Adhesion Targeting—Given the differences in molecular exchange between wild-type NEDD9, NEDD9ΔCT, NEDD9.DFDF, and NEDD9ΔSH2BD, we questioned whether these differences might reflect differential interaction with NEDD9 partner molecules. As FAK is a well established partner of NEDD9 (13), FAK interaction with each of the NEDD9 constructs was assessed by co-immunoprecipitation. This revealed that NEDD9, NEDD9.DFDF, and NEDD9ΔSH2BD show equivalent and robust interaction with FAK (Fig. 5A). By contrast, considerably less FAK co-immunoprecipitates with NEDD9ΔCT. Thus we next confirmed that FAK is required for NEDD9 targeting to focal adhesions by analyzing GFP.NEDD9 subcellular distribution in FAK−/− fibroblasts. This revealed that GFP.NEDD9 does not target to focal adhesions in the absence of FAK expression (Fig. 5B). Importantly, exogenous expression of FAK restores NEDD9 targeting to focal adhesions (Fig. 5B). Finally, FRAP analysis of NEDD9ΔCT in the FAK−/− fibroblasts revealed that in the absence of FAK, this truncated protein now diffuses freely in the region of focal adhesions, with rates of recovery 3-fold faster than those seen in the NEDD9+/− fibroblasts that endogenously express FAK (Fig. 5, C and D). Based on these findings we suggest that the interaction
between NEDD9ΔCT and FAK may attenuate NEDD9ΔCT dynamics but requires the NEDD9 C-terminal domain to anchor NEDD9 at the focal adhesions.

**NEDD9 SH2BD Affects Focal Adhesion Signaling**—Next we questioned the effect of the altered rates of molecular exchange on focal adhesion morphology and signaling. Comparison of
the total number of focal adhesions revealed that neither NEDD9.DFDF nor NEDD9.H9004/SH2BD altered the number of focal adhesions per cell (Fig. 6, A and B). However, focal adhesions in cells expressing NEDD9.H9004/SH2BD were significantly larger (Fig. 6 C). To investigate whether expression of the NEDD9 mutant constructs altered focal adhesion signaling, we analyzed paxillin phosphorylation at focal adhesions. Ratio imaging of focal adhesions co-immunostained with antibodies to total paxillin and paxillin phosphorylated at Tyr(P)-118 suggested that paxillin phosphorylation at focal adhesions is reduced in cells expressing NEDD9.DFDF and NEDD9.H9004/SH2BD (Fig. 6A). Semiquantitative measurement of the ratio of phosphorylated paxillin at focal adhesions confirmed this observation (Fig. 6D).

**DISCUSSION**

NEDD9 localization to focal adhesions is key to the function of this protein in cell migration; however, the molecular regulation of NEDD9 exchange at focal adhesions has not been previously explored. In the present study we found that Src kinase activity is required to slow NEDD9 exchange at focal adhesions and deletion of the NEDD9 domain that is targeted by Src significantly increases the speed of NEDD9 exchange. We, therefore, propose that Src phosphorylation of NEDD9 is an important determinant of NEDD9 residency time at focal adhesions.

Although it has long been known that focal adhesions are regulated via assembly and turnover, the constant molecular exchange between focal adhesion-associated molecules and their cytoplasmic counterparts is a more recently recognized control mechanism (2). There is precedence for signaling-dependent regulation of focal adhesion residency time. Calcium flux slows FAK recovery, thus prolonging FAK association at focal adhesions (28), whereas phosphorylation of the FAK autophosphorylation site (Tyr(P)-397) was sufficient to increase residency time (29). We have now shown that Src regulates the
The rate of protein recovery at the focal adhesions depends on the stability of the interaction with proteins in the focal adhesions. Because Src is a major regulator of focal adhesion disassembly, including directly regulating FAK activity and actomyosin contractility, it was necessary to test whether the effects on NEDD9 were either direct or secondary to other Src targets. Importantly, the demonstration that the NEDD9 SH2BD is required to regulate the rate of NEDD9 molecular exchange confirmed that the effects seen were direct.

Focal adhesion components examined to date exist in at least two states: an immobile fraction and a fraction that exchanges between the cytoplasm and the focal adhesion. Recent reports have also suggested a third behavior where attenuated diffusion of focal adhesion components in the juxtamembrane region close to the focal adhesions serves as a pool of molecules for exchange with the focal adhesions (27). Proteins that freely diffuse at focal adhesions should have a mobile fraction of 100%, and a reduced mobile fraction suggests strong binding to fixed components (30). We found that NEDD9 has a relatively high mobile fraction, with ~80% of the total pool recovered post-bleaching. The related Cas family protein p130Cas (26) has also been shown to have a high mobile fraction as compared for example to paxillin (31). Interestingly, paxillin contains multiple LIM domains that mediate direct binding to actin (32). Conceivably, the reduced mobility of paxillin at focal adhesions may be due to paxillin binding to a stable actin core. By contrast, NEDD9 and p130Cas may not interact with stable structural elements of the focal adhesions and hence have relatively high mobile fractions. Notably, the magnitude of difference between the mobile fractions of the NEDD9 mutant proteins was very small, suggesting that the NEDD9 SBD and SH2BD have limited impact on the total fraction of mobile NEDD9 at focal adhesions.

The present study expands the previously ascribed role for Src kinase in focal adhesion turnover (25) to a novel role in regulating molecule exchange rates at focal adhesions. Given the large array of Src substrates at focal adhesions (34), we suggest that this may prove to be a more general function of Src at focal adhesions, identifying a new biological role for this important enzyme (7, 12, 16–18).

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