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SNARE-mediated membrane traffic is required for focal adhesion kinase signaling and Src-regulated focal adhesion turnover

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ABSTRACT

Integrin signaling is central to cell growth and differentiation, and critical for the processes of apoptosis, cell migration and wound repair. Previous research has demonstrated a requirement for SNARE-dependent membrane traffic in integrin trafficking, as well as cell adhesion and migration. The goal of the present research was to ascertain whether SNARE-dependent membrane trafficking is required specifically for integrin-mediated signaling. Membrane traffic was inhibited in Chinese hamster ovary cells by expression of dominant-negative (E329Q) *N*-ethylmaleimide-sensitive fusion protein (NSF) or a truncated form of the SNARE SNAP23. Integrin signaling was monitored as cells were plated on fibronectin under serum-free conditions. E329Q-NSF expression inhibited phosphorylation of focal adhesion kinase (FAK) on Tyr397 at early time points of adhesion. Phosphorylation of FAK on Tyr576, Tyr861 and Tyr925 was also impaired by expression of E329Q-NSF or truncated SNAP23, as was trafficking, localization and activation of Src and its interaction with FAK. Decreased FAK–Src interaction coincided with reduced Rac activation, decreased focal adhesion turnover, reduced Akt phosphorylation and lower phosphatidylinositol 3,4,5-trisphosphate levels in the cell periphery. Over-expression of plasma membrane-targeted Src or phosphatidylinositol 3-kinase (PI3K) rescued cell spreading and focal adhesion turnover. The results suggest that SNARE-dependent trafficking is required for integrin signaling through a FAK/Src/PI3K-dependent pathway.

1. Introduction

Adhesive interactions between cells and extracellular matrix (ECM) components such as fibronectin (FN) are mainly dependent on integrins which serve to link the ECM to intracellular signaling and the cytoskeleton. This link is critical for the initiation and maintenance of a variety of signaling pathways leading to cell motility, differentiation and survival. Upon engagement, integrins cluster and assemble a variety of intracellular proteins at focal adhesions (FA). An essential protein for FA assembly, turnover and signaling is a nonreceptor protein-tyrosine kinase, focal adhesion kinase (FAK), which binds and phosphorylates a variety of signaling and adaptor proteins, such as Src, p130Cas, paxillin and phosphatidylinositol 3-kinase (PI3K). Several lines of investigation indicate that it is the FAK–Src complex that controls FA turnover, cell morphology, motility and survival [1,2]. As FAK is activated upon clustering at FAs, it autophosphorylates Tyr397, creating a binding site for SH2 domains of several proteins, in particular Src [3]. Src binding to FAK facilitates the transphosphorylation and activation of Src on Tyr416 and multiple FAK tyrosine residues. Phosphorylation of FAK increases FAK kinase activity and binding of other signaling and/or adaptor proteins, eventually leading to the formation of Rac-dependent lamellipodia and turnover of FAs through an as yet ill-defined mechanism.

Src is a myristoylated, non-palmitoylated member of the Src-family of protein-tyrosine kinases that localizes to the plasma membrane and to a perinuclear compartment [4]. Evidence indicates that inactive Src localizes to a perinuclear compartment, and translocates to the plasma membrane and FAs where it is activated. In cells expressing a dominant-negative Rab11 protein, Src is retained in an inactive state in a perinuclear compartment [5]. This localization of Src appears to be separate from emerging roles for Src in regulating cargo sorting and actin polymerization during vesicular transport [6,7].

During intracellular membrane trafficking, vesicle movement between compartments of the cell is controlled by the SNARE (soluble NSF [N-ethylmaleimide-sensitive factor] attachment protein receptor) family of membrane proteins. SNAREs can be classified into two sub-families: vesicle-SNAREs (v-SNAREs, found on the vesicles) and target-SNAREs (t-SNAREs, found on target membranes). Upon trafficking of a vesicle to a target membrane, v-SNAREs and t-SNAREs form cytoplasmic coiled-coil trans-SNARE complexes bridging the two membranes bringing them into proximity and facilitating fusion.

Abbreviations: CHO, Chinese hamster ovary; ECM, extracellular matrix; FN, fibronectin; FA, focal adhesions; FAK, focal adhesion kinase; NSF, *N*-ethylmaleimide-sensitive fusion protein; PI3K, phosphatidylinositol 3-kinase; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; SNARE, soluble NSF attachment protein receptor

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Following fusion, the cis-SNARE complexes are disassembled by the ATPase, NSF, and its adaptor protein α-SNAP (soluble NSF attachment protein) allowing recycling of SNAREs. NSF function is required to disassemble SNARE complexes, thus inhibition of NSF can act as a global inhibitor of membrane trafficking [8].

Previous work from our laboratory has shown that SNARE-mediated membrane traffic is required for integrin trafficking, lamellipodium extension, cell migration, and maintenance of normal FAs [9–11]. These are phenotypes seen in $FAK^{-/-}$ cells suggesting that membrane traffic may have a role in integrin–FAK signaling. In the present study, we report that inhibition of SNARE-mediated membrane traffic obstructs Src translocation from a Rab11-containing recycling compartment to the plasma membrane, perturbing interaction with FAK and inhibiting FAK–Src dependent signaling. This results in reduced cell spreading and FA turnover. Expression of a plasma membrane-targeted Src and p110-subunit of PI3K rescues cell spreading and FA turnover. Our findings suggest that SNARE-mediated traffic plays a key role in signaling downstream of integrins and modulates cell motility principally by controlling a FAK–Src signaling complex.

2. Experimental procedures

2.1. Reagents and cDNA constructs

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Nepean, ON) unless otherwise indicated. Antibodies were obtained against NSF from Stressgen Biotechnologies (Victoria, BC), Rab11, phosphoTyr397-FAK and mouse anti-FAK (F15020) from BD Biosciences (Mississauga, ON), rabbit anti-FAK from Abcam (ab40794; Cambridge, MA), phosphoTyr576-FAK from Novus Biologicals (Littleton, CO), phosphoTyr925-FAK and phosphoSer473-Akt from Cell Signaling Technology, Inc. (Boston, MA), phosphoTyr418-Src, phosphoTyr861-FAK and Src from Applied Biological Materials, Inc. (Richmond, BC), α-tubulin from Invitrogen, Co. (A11126; Mississauga, ON), and Rac from Cytoskeleton, Inc. (Denver, CO). The pcDNA3.1, pcDNA3.1-SNAP23fl, and pcDNA3.1-$SNAP23^{CΔ9}$, were kind gifts from Dr. W.S. Trimble (Hospital for Sick Children, Toronto, ON). The pEGFP-$SNAP23^{CΔ9}$ and pEGFP-SNAP23fl constructs were generated by inserting the *XhoI*/*EcoRI* fragment from the full-length and truncated pcDNA3.1-SNAP23, respectively, into peGFP-C1. The FLAG-SNAP23 constructs were generated by subcloning the full-length SNAP23 into pcDNA3.1-FLAG, a kind gift from Dr. Nina Jones (University of Guelph, Guelph, Canada). The pERFP-PH-Akt was a kind gift from Dr. M. Skinner (University of Guelph, Guelph, ON). The generation of pcDNA3.1–wild-type NSF and E329Q-NSF constructs is described elsewhere [9]. GFP-FAK was provided by Dr. L.H. Tsai (MIT, Cambridge, MA). GFP-talin was a kind gift of Dr. A. Huttenlocher (University of Wisconsin, Madison, WI). The pEGFP-Rab11 and $Rab11^{S25N}$ constructs were kind gifts of Dr. John Brumell (Hospital for Sick Children, Toronto, ON). The pcDNA3.1-PI3K-CAAX was obtained from Addgene, Inc [Addgene plasmid 12591] [12]. pUSEamp containing wild-type Src and a C-terminal truncated constitutively active Src were kind gifts of Dr. Nina Jones (University of Guelph, Guelph, ON). The membrane-targeted Src-CAAX was generated by PCR from pUSEamp-SrcWT using Src-CAAX forward (5′-ataactcgagatgggcagcaacaagagc-3′), Src-CAAX reverse (5′-ataaaagaattctcacataactgtacaccttgtccttgataggttctccccgggctggtact-3′) primers followed by cloning into pcDNA3.1(−).

2.2. Cell culture and transfection

Chinese hamster ovary (CHO)-K1 or CHO cells expressing E329Q-NSF under an inducible promoter [CHO-E329Q; TeT-ON, BD Biosciences, Mississauga, ON; described elsewhere [9]] were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum at 37 °C and 5% (v/v) CO_2. Cells were transfected using FuGENE6 transfection reagent following the manufacturer's suggested protocol (Roche Applied Science, Laval, QC) or calcium phosphate-DNA precipitation. DNA complexes were incubated on the cells for 15 h with the SNAP23 and $Rab11^{S25N}$ constructs, 9 h when transfecting with NSF containing plasmids, or 4–6 h prior to induction of E329Q-NSF when using pERFP-PH-Akt or Src constructs (total expression 13–17 h). Co-transfection studies were performed at a 10:1 molar ratio of DNA-to-marker DNA.

2.3. Immunofluorescence assays

To monitor spreading or FA formation, CHO-K1 cells were transfected, lifted and plated onto FN-coated (10 μg/mL—FA formation; 20 μg/mL—lamellipodia extension) glass coverslips, washed with PBS and fixed with 4% paraformaldehyde (w/v)/PBS. To monitor Src and PIP_3 localization, CHO-E329Q cells were induced with 2 μg/mL doxycycline or mock treated for 9 h, lifted and plated on FN (20 μg/mL) coated coverslips and allowed to adhere, followed by fixation in ice-cold 0.1% Triton X-100 (v/v), 2% paraformaldehyde (w/v) in PBS at 4 °C for 30 min with shaking. Samples were then permeabilized and blocked before staining with primary and secondary antibody, and/or rhodamine-phalloidin (Invitrogen, Mississauga, ON) followed by washing and mounting. Samples were imaged using a 40× or 63× (NA 1.4) lens on a Leica DM-IRE2 inverted microscope with a Leica TCS SP2 system (Leica, Heidelberg, Germany). Images were captured and 3D reconstructions were performed using Leica Confocal Software package. Lamellipodium extension and FA size were assessed in ImageJ software as described elsewhere [10,11]. For Src trafficking assays, cells adherent for 15 or 60 min were visually scored as having primarily peripheral or perinuclear Src staining.

For live cell imaging, CHO cells were transfected, lifted in serum- and phenol red-free DMEM/F12 (Gibco, Missisauga, ON) supplemented with 15 mM HEPES, pH 7.4, plated on FN-coated coverslips for 90 min. Then the coverslips were inserted in a Series 40 chamber in a QE-1 heated platform (Harvard Apparatus Canada, Saint-Laurent, QC), sealed on top with a coverslip and mounted on the microscope stage. Temperature was maintained at 37 °C with a TC-324B temperature controller (Harvard Apparatus Canada, Saint-Laurent, QC). Images were acquired every 30–60 s for approximately 30 min. Time series were imported into ImageJ software for processing.

2.4. Immunoprecipitation and Western blotting

CHO-E329Q cells were induced with 2 μg/mL doxycycline or mock treated (DMSO alone) for 9 h, or co-transfected with GFP-FAK and pcDNA3.1-SNAP23fl or pcDNA3.1-$SNAP23^{CΔ9}$ for 15 h, the last hour of which the cells were serum-starved. Cells were lifted and equal numbers were held in suspension for 15 min or plated on FN for the indicated times. For phosphoprotein probing, cells were lysed *in situ* in SDS sample buffer containing 20 μg/mL DNAase and equal volumes of lysate were analyzed by Western blotting. For immunoprecipitations, cells were harvested and lysed in 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 2 mM sodium fluoride, 2 mM sodium vanadate (lysis buffer) and Sigma complete protease inhibitor cocktail. Lysates were pre-cleared and supernatants containing 1 mg of protein were incubated with FAK (BD Biosciences, Mississauga, ON) or GFP antibody (Abcam, Cambridge, MA) immobilized on Protein G-Sepharose 4B beads (Sigma, Oakville, ON) for overnight. The beads were washed three times with lysis buffer before elution with hot 2× Laemmli buffer for SDS-PAGE/Western blot analysis of Src. Antibody complexes were detected by ECL Plus chemiluminescence (GE Healthcare, Baie d'Urfe, QC). Where appropriate membranes were stripped (62.5 mM Tris–HCl, pH 6.8, 2% SDS and 100 mM β-mercaptoethanol) for 15 min at 50 °C, washed extensively and re-probed for FAK.

2.5. Rac1 activation assay

RacGTP pull-down and analysis were done using a kit from Cytoskeleton, Inc. (BK035; Denver, CO) using the manufacturer's protocol.

2.6. Cell fractionation

CHO-E329Q cells were induced with 2 μg/mL doxycycline or mock treated for 9 h, the last hour of which the cells were serum-starved. Cells were lifted held in suspension for 15 min or plated on FN for the indicated times. Cells were lysed *in situ* in homogenization buffer (50 mM HEPES, pH 7.4, 50 mM NaCl, 1 mM $MgCl_2$, 2 mM EDTA, 1 mM DTT, Sigma protease inhibitor cocktail), lysates were collected with scraping on ice and homogenized by 6–8 passage through a 21 gauge needle. Lysates were centrifuged at 500 *g* for 10 min to remove unbroken cells then the supernatant centrifuged for 20 min at 500,000 *g* to obtain a cytosolic fraction and a membrane-pellet fraction. The membrane fraction was resuspended in Laemmli sample buffer and samples were analyzed by SDS-PAGE–western blot for Rac1, Src or β1-integrin.

3. Results

3.1. Inhibition of SNARE-mediated membrane traffic impairs focal adhesion turnover

Previously, we have shown that inhibition of SNARE-mediated membrane trafficking with dominant-negative NSF (E329Q-NSF) and truncated SNAP23 ($SN23^{C\Delta9}$) impairs CHO spreading and causes the formation of enlarged focal adhesions (FA) [11]. This was demonstrated using GFP-paxillin to monitor FAs and here we expand upon this approach using GFP-FAK and GFP-talin. CHO cells were co-transfected with either GFP-FAK or GFP-talin, along with wild-type NSF (wtNSF), E329Q-NSF, SNAP23 ($SN23^{FL}$), truncated $SN23^{C\Delta9}$ or control vector. FA sized was then measured following adhesion to FN for 2 h. As previously found with paxillin, inhibition of membrane trafficking with E329Q-NSF and $SN23^{C\Delta9}$ resulted in GFP-FAK- and GFP-talin-containing FAs that were between 40% and 70% larger than those found in control cells (Fig. 1A). Expression of wtNSF and $SN23^{FL}$ had little effect on FA size (Fig. 1A). Similar results were seen when endogenous proteins were stained by indirect immunofluorescence (data not shown). To determine if these FAs were larger because of a defect in FA turnover, FA dynamics were monitored in real time following adhesion to FN. GFP-paxillin was used for imaging FAs in live cells because of the clear signal it provides when incorporated into FAs. In control cells expressing wtNSF, GFP-paxillin containing FAs form, disassemble and appear to move along stress fibers—indicative of robust FA turnover (Fig. 1B; Supplementary video 1). In cells expressing E329Q-NSF, FAs assemble as cells adhere, but rarely disassemble or move along stress fibers (Fig. 1B; Supplementary video 2).

3.2. SNARE-mediated traffic is required for FAK phosphorylation and interaction with Src

The observed inhibition of cell spreading and enlargement of FAs (Section 3.1) may be explained by a defect in integrin signaling. In cases where key FA proteins, such as FAK, and Src-family kinases, have been knocked out or expression knocked down, cells have been shown to spread and migrate poorly, and to contain enlarged FAs due to a lack of FA turnover [13–15]. In several studies it has been shown that the association of FAK and Src, and the subsequent phosphorylation of FAK on several key residues, are crucial to maintaining cell motility and FA turnover [16]. Biochemical analyses of FAK and proteins with which FAK interacts are inefficient using mixed populations of cells obtained through transient transfection. To avoid this inefficiency, phosphorylation of FAK and FAK binding of Src were assessed in CHO cells that contain a stably integrated E329Q-NSF under the control of a doxycycline-inducible promoter (TeT-ON system). These cells have been described previously (12) and allow acute inhibition of SNARE-mediate membrane traffic, uniformly in a population of cells. The cells were induced to express E329Q-NSF (+), or mock treated (−), serum-starved for 1 h (to minimize growth factor-induced signaling), lifted and plated on FN for the times indicated in Fig. 2A. Cells held in suspension for 15 min served as negative controls (Susp). In cells not treated with doxycycline, phosphorylation on Tyr416 of Src and Tyr397 of FAK could be detected within 15 min of adhesion to FN. Phosphorylation at these sites rose modestly during the first 30 min of adhesion, but leveled off by 45 min. In contrast to this, cells expressing E329Q-NSF showed reduced phosphorylation on Tyr416 of Src at all time points and reduced phosphorylation of FAK Tyr397 at 15 and 30 min (Fig. 2A). By 45 min, phosphorylation of FAK Tyr397 had recovered, suggesting that FAK was being incorporated into FAs and that clustering of FA components was inducing FAK autophosphorylation. FAK phosphorylation sites dependent on Src activity [Tyr576 [17], Tyr861 [18] and Tyr925 [19]], however, showed decreased phosphorylation at all points during adhesion (Fig. 2A). These observations were made repeatedly and quantification of replicate western blots is shown in Fig. 2B.

Expression of dominant-negative NSF is predicted to cause an inhibition of all SNARE-mediated membrane traffic. To more specifically probe the role of SNARE-dependent traffic in FAK signaling, we disrupted the function of SNAP23 using $SNAP23^{C\Delta9}$ as described for Fig. 1. CHO-K1 cells were co-transfected with the $SNAP23^{C\Delta9}$ construct and GFP-FAK, and the phosphorylation of FAK was monitored as above. Expression of $SNAP23^{C\Delta9}$ did not influence phosphorylation of FAK Tyr397, but did disrupt phosphorylation of FAK Tyr576, Tyr861 and Tyr925 (Fig. 3). The blot shown in Fig. 3A is representative of several replicates, the quantification of which is shown in Fig. 3B. The results suggest that inhibition of SNARE-mediated traffic, generally with dominant-negative NSF or specifically by inhibiting SNAP23, perturbs ECM-induced phosphorylation of FAK.

The effect that inhibiting NSF has on the phosphorylation of Src and FAK (Fig. 2) suggests that Src may not be binding FAK when membrane traffic is inhibited. To test this, FAK was immunoprecipitated from control cells and E329Q-NSF-expressing cells, either held in suspension or allowed to adhere to FN for 15 and 90 min (Fig. 4A). In control cells, Src precipitated with FAK under adherent conditions, but did not do so when cells were in suspension—as reported previously [3,20]. In E329Q-NSF-expressing cells, Src failed to efficiently precipitate with FAK when cells were adherent or suspended. Similarly, when $SNAP23^{C\Delta9}$ was co-expressed with GFP-FAK, little Src co-precipitated with GFP-FAK when cells were adherent or suspended (Fig. 4B). Taken together, these results indicate that inhibition of SNARE-mediated membrane trafficking in CHO cells prevents FAK–Src interaction.

3.3. Inhibition of SNARE-mediated traffic traps Src in a Rab11 compartment

Src is membrane associated via a cotranslational myristoylation at Gly2 and it is generally thought to be located on the cytoplasmic face of the plasma membrane where it is free to interact with FAK, but there is significant evidence that Src can also be found on a variety of intracellular membranes, in particular the recycling endosome [5]. These intracellular compartments may serve as a reservoir from which Src is trafficked to the plasma membrane. Src localization during spreading was examined to determine if E329Q-NSF was trapping Src in an intracellular compartment. It was determined that at the onset of adhesion (15 min after plating on FN) a large component of membrane-associated Src localized to a perinuclear Rab11-containing compartment. This was the case in untreated cells (Fig. 5M), wtNSF-expressing cells and E329Q-NSF-expressing cells

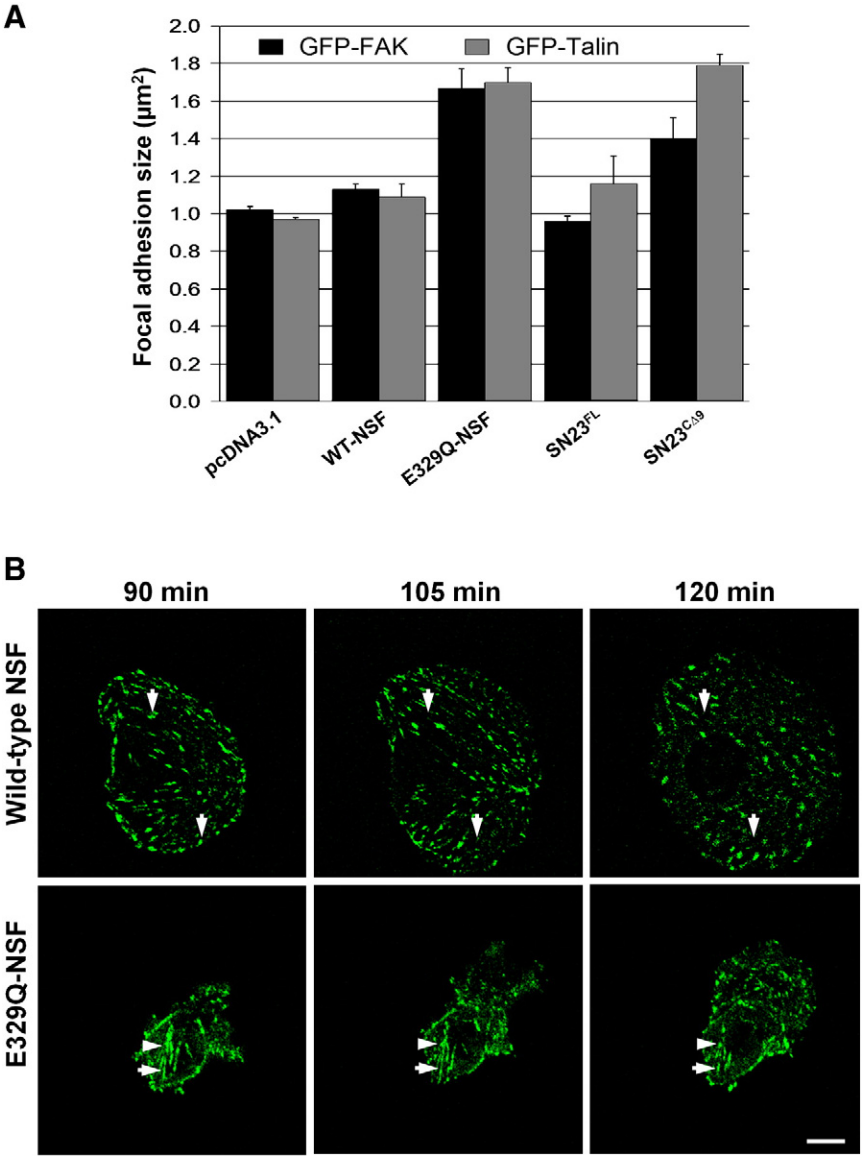

Fig. 1. Inhibition of SNARE-mediated membrane traffic inhibits focal adhesion turnover. A. CHO-K1 cells were transiently co-transfected with either empty vector (*pcDNA3.1*), wild-type NSF (*WT-NSF*), dominant-negative NSF (*E329Q-NSF*), full-length SNAP23 ($SN23^{FL}$) or truncated SNAP23 ($SN23^{C\Delta9}$) along with either GFP-tagged FAK (*GFP-FAK*) or GFP-tagged talin (*GFP-talin*) for 9 h, lifted and plated on fibronectin (10 μg/mL) for 2 h. Cells were fixed, imaged and focal adhesion size was measured using ImageJ software. Results are the means ± SEM of at least 3 separate experiments in which at least 10 cells were analyzed. B. CHO-K1 cells were transiently co-transfected with GFP-paxillin and either wild-type NSF or dominant-negative NSF for 9 h, plated on FN-coated coverslips (10 μg/mL) for 1.5 h, then FAs containing GFP-paxillin were imaged every 30 s for 30 min. Time series were imported into ImageJ for processing. Images are representative of at least 5 separate experiments. Arrowheads in the top row point to FAs, present at 90 min that subsequently disappear. Arrowheads in the bottom row point to examples of persistent FAs. Scale bar = 10 μm.

(Fig. 5). In wtNSF-expressing control cells, 15 min after plating on FN, Src is found both in a central Rab11-containing compartment and in locations in the cell periphery (Fig. 5A–C). As control cells spread (60 min time point), Src redistributes from the central compartment to predominantly the cell periphery (Fig. 5G–I). In E329Q-NSF-expressing cells, much of the Src remains in the central Rab11-containing compartment, even at 60 min (Fig. 5J–L). Similar results were observed when SNAP23 function was inhibited by expression of $SNAP23^{C\Delta9}$ (micrographs not shown, quantification shown in Fig. 5M). Fig. 5M shows results from replicate experiments where the percentage of cells containing Src in the perinuclear compartment has been quantified in fluorescent micrographs. These differences in Src localization were not the result of dissimilar amounts of Src associated with cellular membranes in wild-type- and E329Q-NSF-expressing cells. As shown in Supplementary Fig. 1, there were no significant differences in the amount of membrane-bound Src between control and E329Q-NSF-expressing cells.

To determine if the trafficking of Src out of the Rab11-containing compartment was dependent on Rab11 function, we examined trafficking of Src in cells transfected with a dominant-negative form of Rab11 ($Rab11^{S25N}$). Similarly to E329Q-NSF, expression of $Rab11^{S25N}$ inhibited trafficking of Src out of the perinuclear compartment (Fig. 5M), while wild-type Rab11 had no effect (data not shown). This effect is consistent with previously published studies of the Rab11-dependence of Src trafficking [5]. These findings suggest that when SNARE-mediated membrane trafficking is inhibited Src cannot transit from the Rab11-containing endosome to participate in integrin-dependent signaling in the cell periphery.

3.4. Inhibition of SNARE-mediated membrane trafficking impairs FAK–Src dependent signaling

FAK–Src signaling during adhesion has been linked to the activation of several signaling pathways involved in cell spreading,

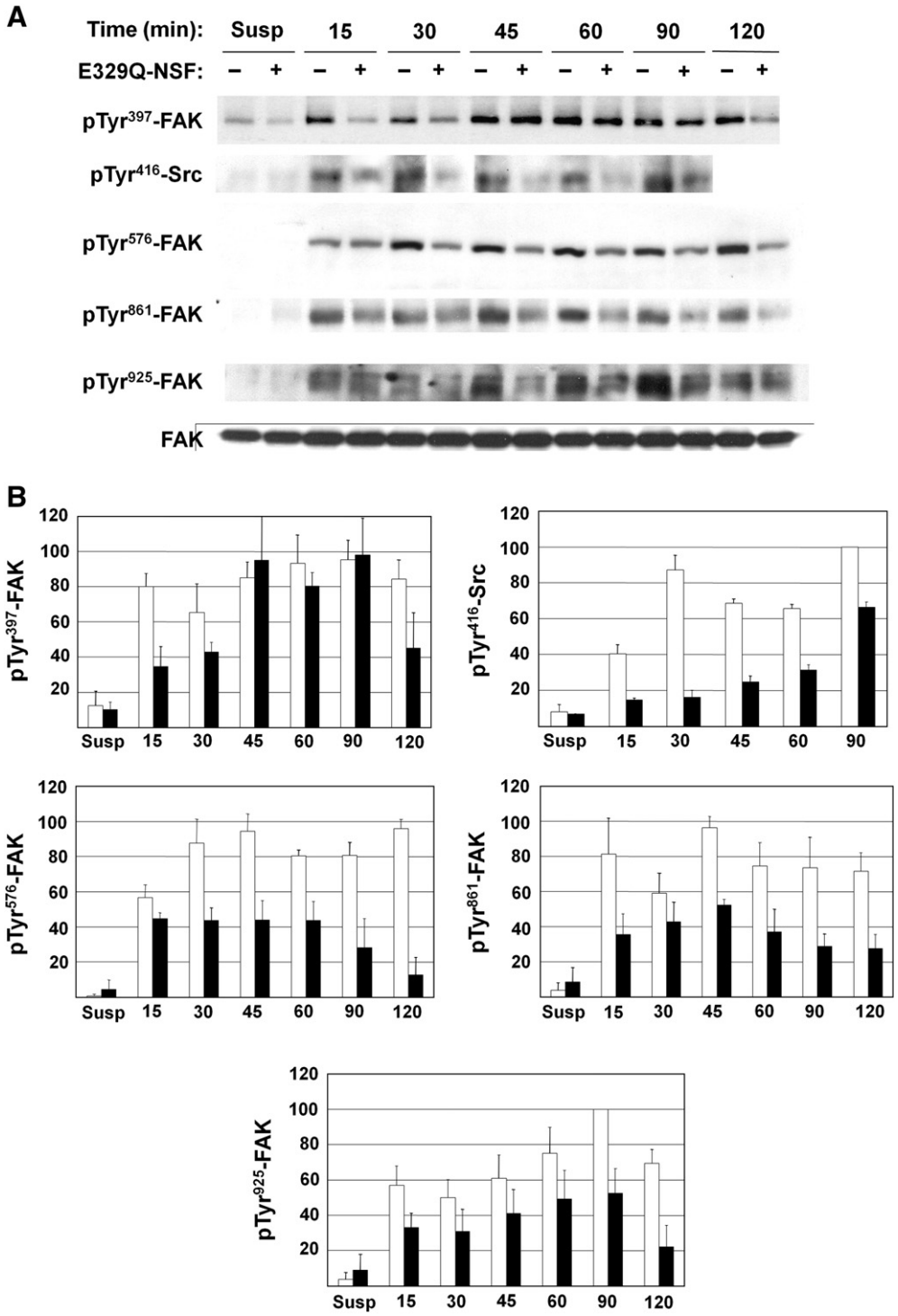

Fig. 2. E329Q-NSF inhibits phosphorylation of FAK and Src. A. CHO-E329Q cells were induced for 9 h (+) or mock treated (−) and serum-starved for the last hour, before being lifted, held in suspension for 15 min (*Susp*) or plated on FN for the times indicated in the top row (which apply to all blots). Cells were lysed *in situ* using SDS sample buffer and analyzed for the indicated phosphoproteins by SDS-PAGE and Western blot with the indicated antibodies. FAK loading control is at the bottom. B. Blots were scanned and the intensities of indicated phosphoproteins were quantified using ImageJ software. Data are presented as percent of maximal response. Mean ± SD from at least 3 independent experiments is shown. *White bars* are data from mock treated CHO-E329Q cells and *black bars* are data from induced cells.

motility and FA turnover [2]. For example, the FAK–Src complex can recruit and activate PI3K at the plasma membrane [21]; Src binding and phosphorylation of Tyr861-FAK lead to recruitment and phosphorylation of p130Cas which initiates Rac signaling via the CrkII–DOCK180–ELMO complex [22,23]. Having already shown that SNARE-mediated membrane trafficking is required for cell spreading, migration and FA turnover [9–11], and that this is possibly due to impaired FAK–Src complex formation, PI3K and Rac activation were assessed, as these are key mediators of cell motility. PI3K activity and PIP_3 production were monitored by the phosphorylation of protein kinase B (Akt) on Ser473, a well documented PI3K-dependent event [24]. Initial adhesion to FN (15 and 30 min) rapidly induced Akt phosphorylation in both control and mutant NSF-expressing cells (Fig. 6A). As the cells adhered, Akt phosphorylation diminished, but this occurred more rapidly in cells expressing E329Q-NSF. Next the location of PIP_3 was examined by expressing the PH domain of Akt

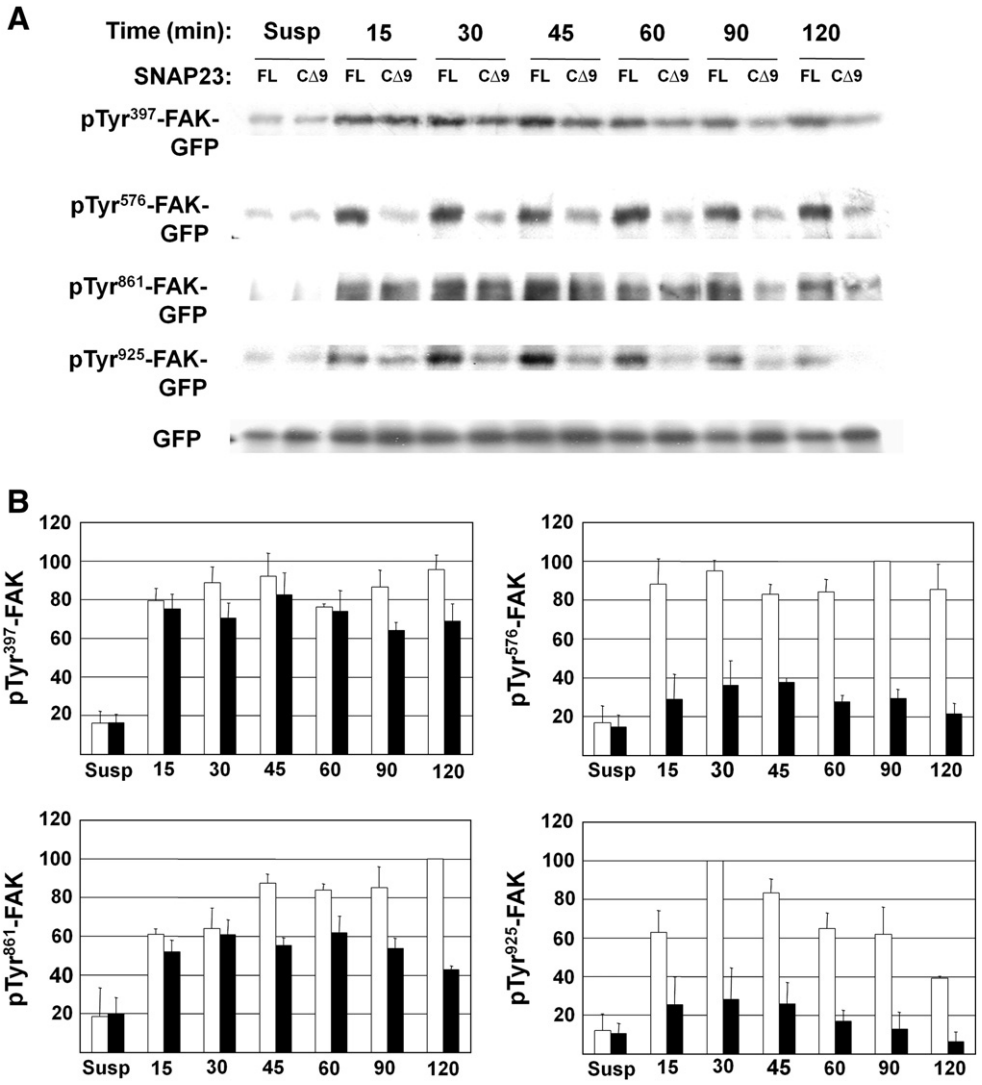

Fig. 3. SNAP23^{CΔ9} inhibits phosphorylation of FAK and Src. A. CHO-K1 cells were co-transfected with GFP-FAK and full-length SNAP23 (*FL*) or truncated SNAP23 (*CΔ9*) (10:1 molar ratio, respectively) for 15 h and serum-starved for the last hour, before being lifted, held in suspension for 15 min (*Susp*) or plated on FN for the times indicated in the top row (which apply to all blots). Cells were lysed *in situ* using SDS sample buffer and analyzed for the indicated phosphoproteins by SDS-PAGE and Western blot with the indicated antibodies. Bands corresponding to GFP-FAK are shown and a representative blot re-probed for GFP as a loading control is at the bottom. B. Blots were scanned and the intensities of indicated phosphoproteins were quantified using ImageJ software. Data are presented as percent of maximal response. Mean ± SD from at least 3 independent experiments is shown. *White bars* are data from full-length SNAP23 co-expressing cells and *black bars* represent data from truncated SNAP23 co-expressing cells.

tagged with RFP (PH-Akt-RFP). In control cells, PIP_3 was found primarily in the periphery, at the edge of the cell, with more diffuse staining near the center of the cell (Fig. 6B). Detectable PIP_3 in the cell periphery noticeably diminished between 45 and 90 min of cell spreading (Fig. 6C–D), coinciding with the decreased Akt phosphorylation observed over this period (Fig. 6A). In E329Q-NSF-expressing cells, PIP_3 localization was more diffuse with no significant accumulation at the peripheral edge of the cell (Fig. 6E). Consistent with reduced Akt phosphorylation, detectable PIP_3 at the cell periphery declined more rapidly in E329Q-NSF-expressing cells than in control cells (Fig. 6F–G). These findings support the notion that SNARE-mediated membrane trafficking of Src is required for efficient activation and/or targeting of PI3K.

During adhesion, Rac activity was monitored both by pull-down of RacGTP with the CRIB-domain from PAK and by assessing membrane association of Rac. As shown previously, Rac was rapidly loaded (15 min time point) with GTP upon adhesion to FN in control cells and this decreased as they spread [25] (Fig. 7A). In E329Q-NSF-expressing cells, the level of RacGTP was significantly reduced. This phenotype was also observed when membrane-associated Rac was examined, with less Rac bound to membranes in E329Q-NSF-expressing cells compared to controls (Fig. 7B).

3.5. Membrane-targeted Src-CAAX and PI3K-CAAX rescue cell spreading and focal adhesion turnover in cells with impaired SNARE function

We have previously attempted to restore cell spreading and normal FA size in cells with impaired SNARE-mediated traffic using constitutively active Rac1-Q61L, dominant-negative RhoA-T19N, and by inhibiting ROCK with Y27632 [11]. In these cases, Rac1-Q61L increased cell spreading, but did not restore normal FA size; the ROCK inhibitor disrupted FA formation and/or promoted turnover, but did not restore lamellipodium formation; RhoA-T19N had no effect. Having shown here that SNARE-dependent trafficking is required for FAK–Src complex activation, we sought to reverse the effects of E329Q-NSF by modulating signaling more proximal to integrins. Initially, we attempted to restore cell spreading and FA turnover by over-expressing wild-type (SrcWT) and a constitutively active C-terminal deletion of Src (SrcCA) with E329Q-NSF; however, while SrcCA did increase spreading when expressed with wtNSF, both SrcWT and SrcCA failed to rescue spreading

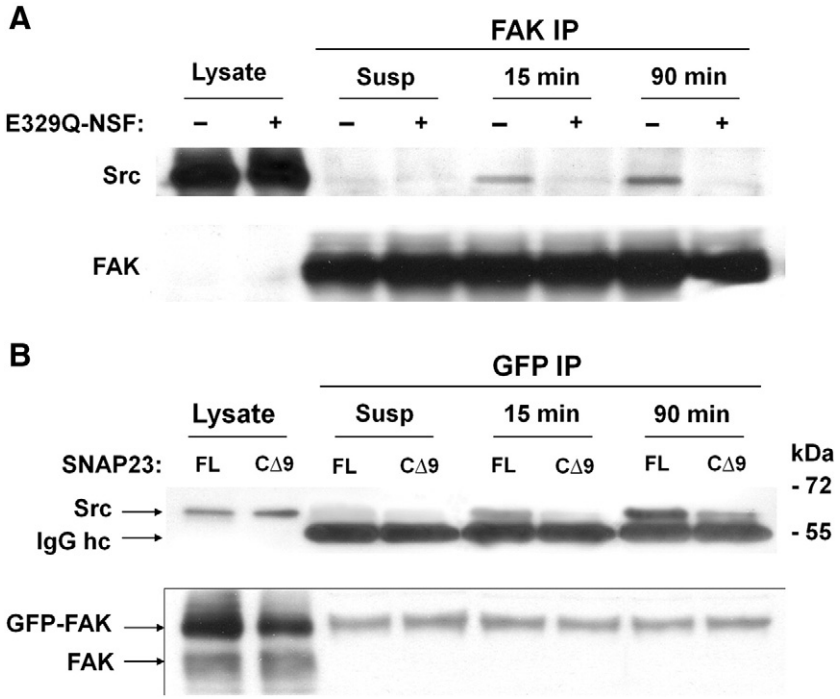

Fig. 4. Inhibition of SNARE-mediated membrane traffic inhibits the interaction of FAK and Src. A. CHO-E329Q cells were induced for 9 h (+) or mock treated (−) and serum-starved for the last hour, before being lifted, held in suspension for 15 min (*Susp*) or plated on FN for the indicated times. Cell lysates were then prepared *in situ*, pre-cleared and FAK was immunoprecipitated with anti-FAK-protein G-Sepharose beads. Immunoprecipitates were washed and analyzed by SDS-PAGE and Western blots for Src (*upper blot*) followed by stripping and re-probing for FAK (*lower blot*). B. CHO-K1 cells were co-transfected with full-length SNAP23 (*FL*) or truncated SNAP23 (*CΔ9*) along with GFP-FAK (10:1 molar ratio, respectively) for 15 h, serum-starved for the last hour, lifted, and held in suspension for 15 min (*Susp*) or plated on FN for the indicated times. Cell lysates were then prepared *in situ*, pre-cleared and GFP-FAK was immunoprecipitated with anti-GFP-protein G-Sepharose beads. Immunoprecipitates were analyzed by SDS-PAGE and Western blots for Src (*upper blot*) and FAK (*lower blot*). The lower band in the Src blot is the IgG heavy chain (IgG hc).

in cells expressing E329Q-NSF (Fig. 8A). This was likely due to the fact that in E329Q-NSF-expressing cells the majority of membrane-associated SrcWT and SrcCA failed to traffic to the plasma membrane and were trapped in a perinuclear Rab11-containing compartment (data not shown; Supplementary Fig. 2, respectively).

We hypothesized that a form of Src that was constitutively targeted to the plasma membrane would support cell spreading and FA signaling in a manner that was not dependent on SNARE-mediated traffic. We therefore generated a Src construct containing the CAAX domain from k-Ras (Src-CAAX), which targets Src to the plasma membrane and transfected this into cells along with E329Q-NSF. Cells expressing Src-CAAX that were also transfected with E329Q-NSF spread similarly to wtNSF-expressing control cells (Fig. 8A). We did observe an increase in cell spreading, above that observed in control cells, as a result of Src-CAAX expression and speculate that this may be attributable to enhanced Src-FAK signaling resulting from increased levels of Src at the plasma membrane. Expression of Src-CAAX also reversed the deficit in cell spreading that was caused by expression of SNAP23^{CΔ9} (Fig. 8B). To determine if Src-CAAX expression could restore normal FA size in cells with impaired SNARE function, we expressed Src-CAAX along with E329Q-NSF or SNAP23^{CΔ9} in cells and measured FA size as in Fig. 1. In cells expressing E329Q-NSF (Fig. 9A) or SNAP23^{CΔ9} (Fig. 9B), Src-CAAX restored FA size to that of control cells. These results suggest that the transport of Src, to the plasma membrane, is required for normal cell spreading and FA signaling.

To test the possibility that PI3K is functioning downstream of SNARE-mediated trafficking of Src, a membrane-targeted catalytic (p110α) subunit of PI3K (PI3K-CAAX) was transiently over-expressed in cells in which E329Q-NSF expression could be induced or which were co-transfected with either SNAP23FL or SNAP23^{CΔ9}. When the PI3K-CAAX construct is over-expressed it serves as a constitutively active PI3K [12]. Using PH-Akt-RFP as a reporter, it was observed that PI3K-CAAX restored PIP_3 production at the cell periphery as expected (data not shown). PI3K-CAAX expression also restored cell spreading (lamellipodium extension) to control levels in cells expressing E329Q-NSF without having a significant effect on spreading in cells expressing wild-type NSF (Fig. 8A). A similar effect of PI3K-CAAX was observed in cells expressing SNAP23^{CΔ9} (Fig. 8B). Consistent with these observations, the size of FAs was reduced to wild-type levels when PI3K-CAAX was expressed in cells also expressing either E329Q-NSF (Fig. 9A) or SNAP23^{CΔ9} (Fig. 9B).

4. Discussion

The present study demonstrates that in CHO-K1 cells SNARE-mediated membrane traffic is needed for efficient translocation of Src from a Rab11-containing compartment to the cell periphery. At the plasma membrane, Src facilitates integrin-dependent signaling leading to cell spreading and FA turnover. When membrane trafficking was inhibited with dominant-negative NSF (E329Q-NSF) or truncated SNAP23 (SNAP23^{cΔ9}), Src trafficking out of the recycling endosome was impeded. This meant that although FAK was recruited to FAs and autophosphorylated on Tyr397, creating a high affinity SH2-binding site, Src was unable to bind FAK. A delay was observed in Tyr397-phosphorylation early during adhesion in cells expressing E329Q-NSF, possibly resulting from reduced delivery of integrins to the plasma membrane as described previously [10,26,27]; however, this delay did not appear to be a primary cause of the defects in spreading and FA turnover observed here. The latter assertion is based on the fact that at later time points of adhesion (after 30 min) phosphorylation of FAK Tyr397 had recovered (Fig. 2), but FAK–Src binding and Akt activation remained impaired. Furthermore, in cells expressing SNAP23^{cΔ9}, phosphorylation of FAK Tyr397 was not perturbed (Fig. 3), yet FAK–Src binding, cell spreading and focal adhesion turnover were decreased.

During cell adhesion, the Src SH2-domain normally binds phosphorylated Tyr397 of FAK, relieving an autoinhibitory conformation in

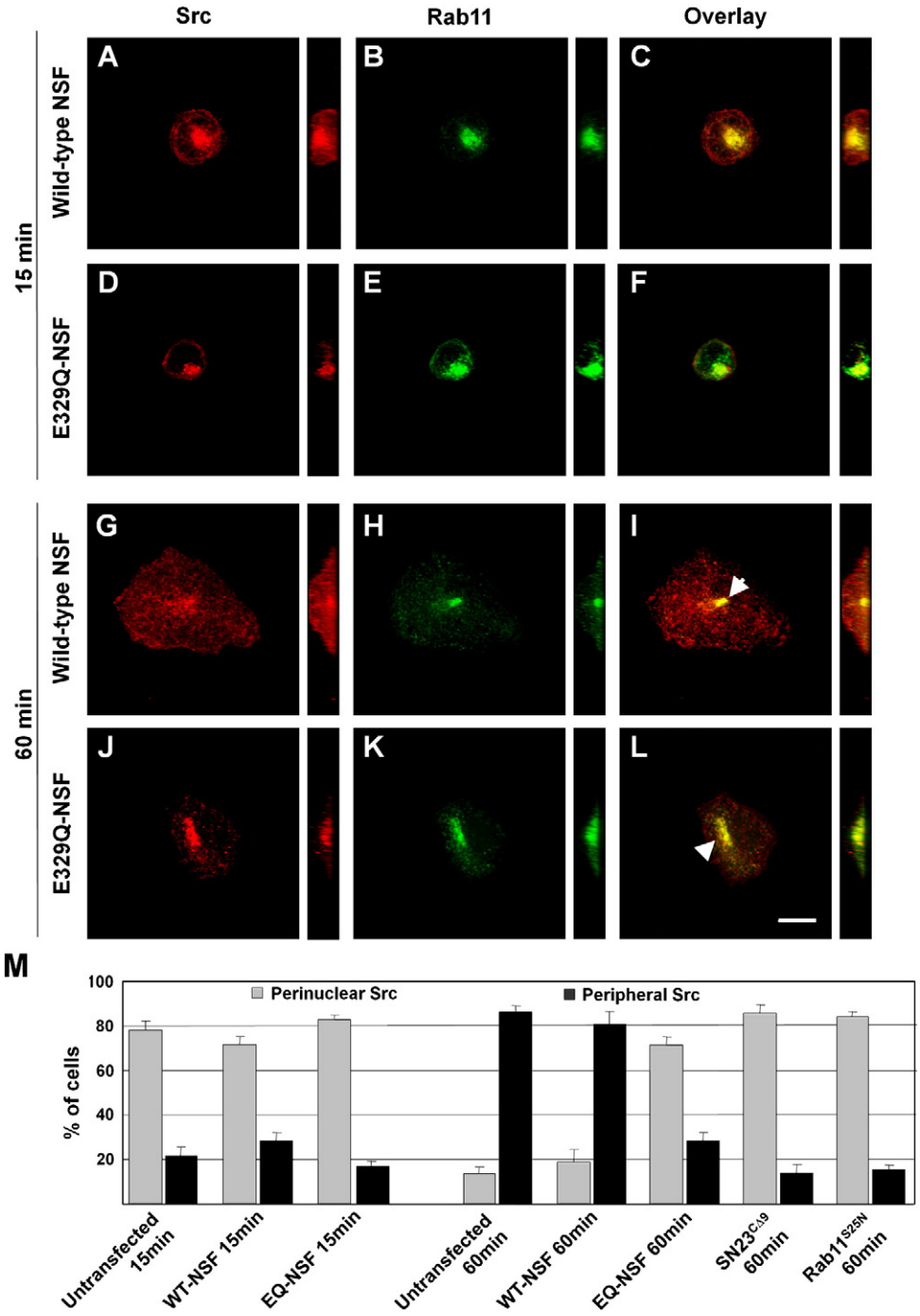

Fig. 5. Inhibition of SNARE-mediated membrane traffic inhibits Src trafficking from a Rab11 compartment. CHO-E329Q cells were mock treated (*Wild-type NSF*; A–C, G–I) or induced with doxycycline (*E329Q-NSF*; D–F, J–L) for 9 h and serum-starved for the last hour, before being lifted, and plated on FN for the indicated times. Cells were fixed in ice-cold 0.1% Triton X-100, 2% paraformaldehyde and stained with anti-Src (*red*; A, D, G, and J) and anti-Rab11 (*green*; B, E, H, and K). Arrowheads point to Src-Rab11-containing compartment in WT-NSF cells (I) and E329Q-NSF cells (L). Note the increased amount of Src in Rab11-containing compartment in L. Scale bar = 10 μm. (M) Cells from the above experiments, as well as untreated cells, and cells where membrane traffic was inhibited with truncated SNAP23 (*SN23*^{cΔ9}) and dominant-negative Rab11 (*Rab11*^{S25N}) were visually scored as having primarily perinuclear Src staining (*light grey bars*), as seen in panel A, or peripheral Src staining (dark grey bars), as seen in panel G. Results are the means ± SEM of at least 3 separate experiments in which at least 50 cells were scored.

Src and allowing phosphorylation of Src on Tyr416 within the activation loop. In cells where membrane traffic was inhibited with E329Q-NSF, Src-Tyr416 phosphorylation was inhibited, consistent with a requirement for Src to be translocated for it to be activated. Furthermore, within the FAK–Src complex, Src has been shown to trans-phosphorylate FAK at Try576/577 within the kinase activation domain, and within the C-terminus at Tyr861 and Tyr925 [1]. These Src-dependent phosphorylations were decreased when membrane trafficking was impaired through inhibition of either NSF or SNAP23. The similarity of the observed effects on FAK signaling resulting from inhibition of membrane traffic in general (E329Q-NSF) or from targeting of SNAP23 (SNAP23^{cΔ9}) suggests that a subset of SNARE-mediated pathways (involving SNAP23) contributes to this signaling mechanism. We are currently working to identify the other SNAREs that collaborate with SNAP23 in this pathway.

The decreased phosphorylation of FAK at Tyr861 and Tyr925 likely has important implications for the observed deficiencies in cell spreading and FA turnover. Phosphorylation of Tyr861 is associated

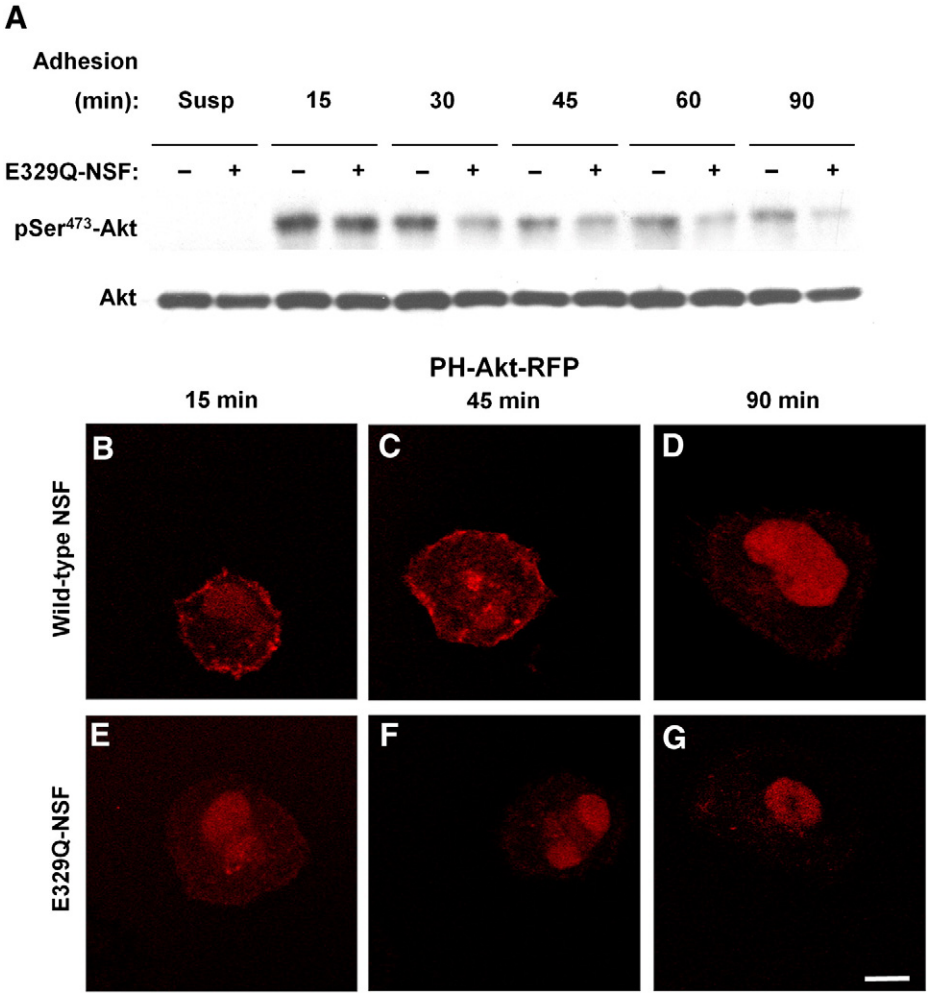

Fig. 6. E329Q-NSF inhibits phosphorylation of Akt and reduces peripheral PIP_3. CHO-E329Q cells were induced for 9 h (+), or mock treated (−), serum-starved for the last hour, lifted and held in suspension for 15 min (*Susp*) or plated on FN for the indicated times. A. Cells were lysed *in situ* using SDS sample buffer and analyzed for phosphorylated Ser473-Akt by SDS-PAGE and Western blot using a phosphospecific antibody (*upper blot*). The membrane was stripped and re-probed for total AKT (*lower blot*). B–G. CHO-E329Q cells were transfected with a RFP-tagged form of the PH domain from Akt (PH-Akt-RFP). After 6 h, cells were induced for 9 h (*E329Q-NSF*; E–G), or mock treated (*Wild-type NSF*; B–D), serum-starved for the last hour, lifted, and plated on FN (20 μg/mL) for the indicated times. Cells were then fixed in ice-cold 0.1% Triton X-100, 2% paraformaldehyde, washed, mounted and imaged by confocal microscopy. Scale bar = 10 μm.

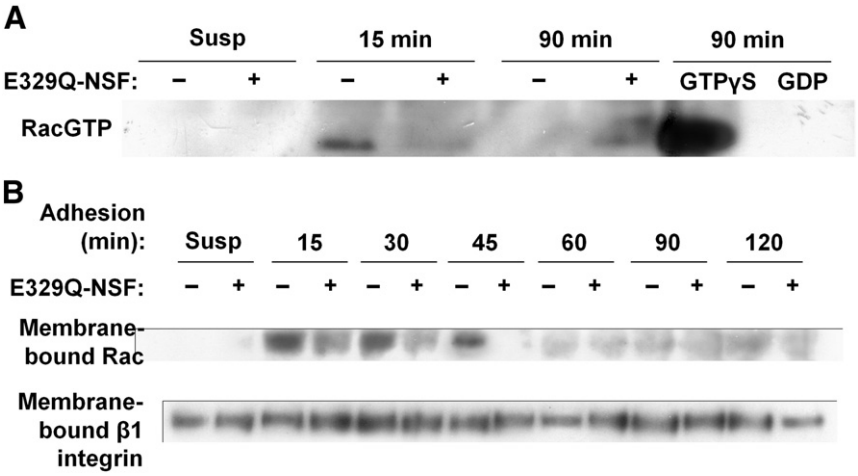

Fig. 7. Inhibition of SNAREs impairs activation of Rac. CHO-E329Q cells were induced for 9 h (+), or mock treated (−), serum-starved for the last hour, lifted and held in suspension for 15 min (*Susp*) or plated on FN for the indicated times. A. Cell lysates were prepared *in situ*, pre-cleared and incubated with CRIB-domain-labeled Sepharose beads. As positive and negative controls, respectively, lysates from cells plated for 90 min were incubated with GTPγS, or GDP, during the RacGTP pull-down. RacGTP was eluted with SDS sample buffer and analyzed by SDS-PAGE and Western blot. B. Cells were lysed in hypotonic lysis buffer, collected by scraping and homogenized. Lysates were cleared to remove unbroken cells and supernatants were ultracentrifuged to collect a membrane fraction. Membrane fractions were analyzed by SDS-PAGE and Western blot for Rac1 and β1-integrin as a loading control.

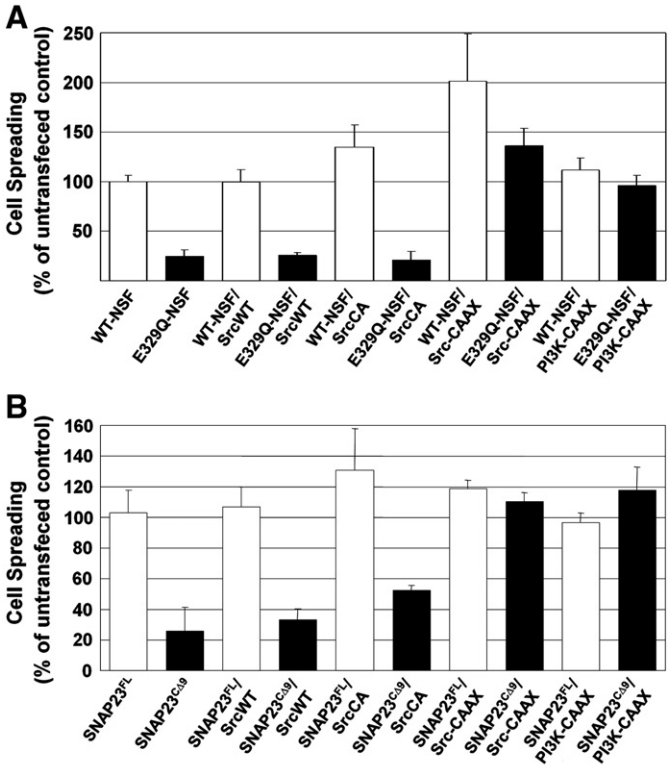

Fig. 8. A plasma membrane-targeted Src or p110α-subunit of PI3K rescue cell spreading in cells expressing E329Q-NSF and truncated SNAP23. A. CHO-K1 cells were transfected with wild-type (*WT-NSF*) or dominant-negative NSF (*E329Q-NSF*), along with control vector, wild-type Src (SrcWT), constitutively active Src (SrcCA), membrane-targeted Src (Src-CAAX) or PI3K-CAAX for 9 h, lifted and plated onto FN-coated coverslips (20 μg/mL) for 90 min, fixed and stained for NSF or Src, and actin to allow assessment of lamellipodium extension. The results are presented as percent of untransfected control cells. B. CHO-K1 cells were co-transfected with full-length GFP-SNAP23 ($SNAP23^{FL}$) or truncated GFP-SNAP23 ($SNAP23^{CΔ9}$), and Src-CAAX, PI3K-CAAX or control vector (1:10 molar ratio, respectively) for 15 h. Cells were lifted and plated on FN and lamellipodium extension analyzed as in A. In A and B, at least 20 randomly selected transfected cells were analyzed per condition for each experiment; means ± SEM of at least three independent experiments are shown.

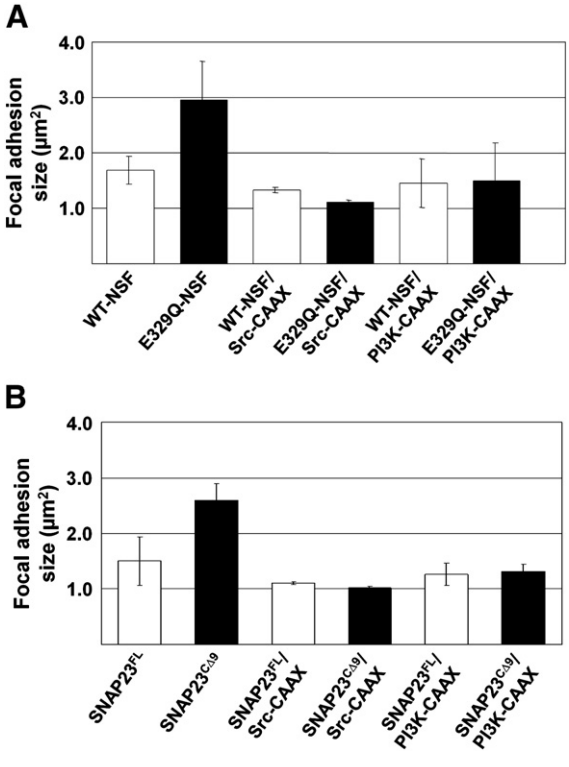

Fig. 9. A plasma membrane-targeted Src or p110α-subunit of PI3K rescue focal adhesion turnover in cells expressing E329Q-NSF. A. CHO-E329Q cells were co-transfected with Src-CAAX, PI3K-CAAX or control vector, along with GFP-paxillin (10:1 molar ratio, respectively), followed by induction of E329Q-NSF (*E329Q-NSF*) or mock treatment (WT-NSF) for 9 h. Cells were lifted and plated on FN (10 μg/mL) for 2 h, followed by fixing, mounting and analysis by confocal microscopy to document and measure focal adhesion size. B. CHO-K1 cells were co-transfected with Src-CAAX, PI3K-CAAX or control vector, and FLAG-SNAP23 full-length ($SNAP23^{FL}$) or FLAG-SNAP23 truncated ($SNAP23^{CΔ9}$), along with GFP-paxillin (10:1:1 molar ratio, respectively) for 15 h. Cells were lifted and plated on FN (10 μg/mL) for 2 h, followed by fixing, staining for FLAG, and analysis of focal adhesions as in A. In A and B, at least 10 randomly selected transfected cells were analyzed per condition for each experiment; means ± SEM of at least three independent experiments are shown.

with increased SH3-dependent binding of p130Cas to FAK, which facilitates tyrosine phosphorylation of p130Cas and recruitment of a CrkII–DOCK180–ELMO complex which regulates Rac1 signaling [22,23]. As was demonstrated during inhibition of membrane traffic, Rac activity and membrane association were reduced. In a previous study, expression of a constitutively active Rac1-Q61L was able to overcome the inhibition in spreading, but FAs remained larger [11]. The present study suggests that the larger FAs result from reduced FA turnover, possibly due to decreased FAK–Tyr925 phosphorylation. Several studies have shown that phosphorylation of FAK at Tyr925 is required for FA turnover. When Tyr925 is phosphorylated, it creates the SH2-binding site for Grb2, which overlaps with the paxillin-binding FAT domain, and consequently binding of Grb2 may displace paxillin from FAK leading to FAK dissociation and FA turnover [28,29]. Tyr925-phosphorylated FAK appears to be excluded from FAs, and expression of a Try925Phe mutant results in larger FAs and less turnover [30]. Similarly, Grb2 may recruit dynamin to FAK leading to a dynamin-dependent FA turnover [31].

Although we did not directly measure FAK kinase activity here, the fact that phosphorylation at both FAK–Tyr576 and Src-Tyr416 were reduced is consistent with reduced cellular FAK activity. It is therefore reasonable to speculate that many signaling pathways dependent on FAK–Src activity, and leading to spreading and FA turnover, might be perturbed. For example, the FAK–Src complex has been implicated in the phosphorylation of paxillin at Tyr31 and Tyr118 which recruits CrkII–DOCK180–ELMO promoting Rac-dependent spreading [32,33]. When these residues in paxillin are mutated to non-phosphorylatable amino acids, the turnover of FAs is inhibited [34]. Also, FAK–Src phosphorylation of GIT2 recruits a GIT2–PIX–PAK complex to paxillin implicated in Rac activation [35,36]. Furthermore, a similar GIT1–PIX–PAK–paxillin complex has been implicated not only in Rac activation, but also FA turnover [37].

The proposal that SNARE-mediated trafficking of Src to the plasma membrane is required for FAK–Src interaction and focal adhesion turnover is supported by the observation that expression of plasma membrane-targeted Src-CAAX reversed the effects that inhibiting SNARE function had on cell spreading and FA size. This notion is underscored by the demonstration that a constitutively active Src (SrcCA) did not rescue spreading. The SrcCA did remain associated with a Rab11-containing compartment in E329Q-NSF-expressing cells (Supplementary Fig. 2), indicating that the translocation of this construct is SNARE-dependent and thus the constitutive activity of SrcCA is mitigated by the fact that it is not properly transported within the cell. Recent studies have documented the translocation of Src from a perinuclear compartment to the plasma membrane under several conditions, including reports showing that this translocation is dependent on membrane traffic [6,38]. In agreement with results shown here, Sandilands et al. [5] found that expression of a dominant-negative mutant of Rab11 inhibited translocation of Src to the plasma membrane [5]. It is not yet known how specific SNARE and Rab family

members are coordinated in the trafficking of Src and work is currently underway to investigate this pathway.

While we have focused on the trafficking of Src here, we cannot exclude the possibility that other protein(s) are trafficked along with Src from a recycling endosome and subsequently influence integrin signaling. Previously, we have shown that inhibition of SNARE-mediated membrane traffic impairs the delivery of α5β1-integrin from a Rab11 compartment similar to the one observed here [10]. It is interesting to speculate that integrin and Src may be trafficking as part of a complex to the plasma membrane and future studies will address this possibility.

The finding that a plasma membrane-targeted p110α-subunit of phosphatidylinositol 3-kinase (PI3K-CAAX) rescued spreading and FA turnover in cells with impaired SNARE function suggests a mechanistic role for SNARE-mediated traffic in regulating FAK–Src signaling. In the context of cell adhesion and spreading, it has been proposed that binding of the SH2 domain of p85-PI3K to Tyr397-phosphorylated FAK results in recruitment and phosphorylation of PI3K [21]. Alternatively, a set of studies has also shown that β1-integrins can activate PI3K in a FAK/Src-independent pathway and this pathway can lead to p130Cas phosphorylation [39,40], indicating that Src is not absolutely required for PI3K activity. Here, we observed that PI3K/Akt can be activated independently of Src trafficking at early time points (Fig. 6A); however, Src or some other SNARE-dependent component is required for sustained activity after 45 min of adhesion and for proper localization of PI3K. Several studies have implicated Src in PI3K/Akt activation [41] and this activation of PI3K can contribute to regulation of actin cytoskeleton remodeling [42,43]. Constitutive targeting of PI3K-CAAX to membranes, as used herein, may bypass this SNARE-dependent form of Src signaling and may lead to Rac activation and spreading via several mechanisms [44]. PI3K/PIP_3 has also been implicated directly in FA turnover, providing another plausible mechanism by which PI3K-CAAX might bypass Src and stimulate FA turnover [45–47].

FAK and Src are central regulators of cell–ECM adhesion. Importantly, both these proteins have well described roles in cancer progression and metastasis, prompting the study of small molecule inhibitors of these proteins in cancer treatment. The results described in the present study reveal that SNARE-mediated membrane traffic has a potent influence over FAK–Src signaling and may contribute to the development of new therapeutic strategies.

Supplementary data to this article can be found online at doi:10.1016/j.bbamcr.2010.09.008.

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