SNARE-mediated membrane traffic modulates RhoA-regulated focal adhesion formation

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Abstract In the present study, we examined the role of soluble NSF attachment protein receptor (SNARE)-mediated membrane traffic in the formation of focal adhesions during cell spreading. CHO-K1 cells expressing a dominant-negative form of *N*-ethylmaleimide-sensitive factor (E329Q-NSF) were unable to spread as well as control cells and they formed focal adhesions (FAs) that were larger than those in control cells. FA formation was impaired in cells transfected with a dominant-negative form of RhoA, but, significantly, not in cells simultaneously expressing dominant-negative NSF. Treatment of E329Q-NSF-expressing cells with the ROCK inhibitor Y-27632 did inhibit FA formation. The results are consistent with a model of cell adhesion in which SNARE-mediated membrane traffic is required for both the elaboration of lamellipodia and the modulation of biochemical signals that control RhoA-mediated FA assembly.

Keywords: Cell adhesion; Focal adhesion; Membrane traffic; *N*-ethylmaleimide sensitive fusion protein; Soluble NSF attachment protein receptor; RhoA

1. Introduction

Cell adhesion to exracellular matrix (ECM) proteins is essential to development and homeostasis in multicellular eukaryotes. Cell–ECM interactions provide physical support for cells and initiate signal transduction pathways that affect cellular activities such as proliferation, differentiation and motility [1,2]. The process of integrin-mediated cell adhesion to ECM involves several steps, including integrin binding of ECM ligand, lamellipodium extension and the formation of actin stress fibers and focal adhesions (FAs). Following integrin engagement and cell attachment, cell spreading is initiated by the activation of the Rho-GTPases Cdc42 and Rac, which promote filipodia and lamellipodia formation, respectively [3,4]. Subsequent to extension of membrane protrusions, signaling through the GTPase RhoA activates ROCK (also known as Rho kinase or ROK) and mDia [5]. ROCK and mDia in turn induce actomyosin-based contractility and actin polymerization, respectively, leading to the clustering of integrins and the formation of focal adhesions and stress fibers [6]. These coordinated events ultimately result in fully spread and firmly adherent cells. As primary sites of cell–ECM contact, FAs are intensively studied and their structure and function as signaling compartments have provided important insight into the molecular mechanism of cell adhesion.

The mechanisms by which FA proteins are localized to nascent cell–ECM contact sites are not completely understood, though there is substantial evidence that integrins are transported to points of attachment via vesicular traffic. For example, it has been shown that integrins are recycled from the trailing edge of a migrating cell to the leading edge [7–9]. It has also been reported that membrane trafficking is involved in the movement of integrin-containing vesicles from a perinuclear region to the bases of forming lamellipodia in fibroblasts [10] and in neutrophils [11]. Furthermore, it has been demonstrated that recycling of integrins from endosomal compartments is necessary for cell adhesion and spreading [12]. Collectively, these results suggest that membrane trafficking is involved in the delivery of adhesive receptors during the establishment of cell–ECM contact in spreading and migrating cells.

During the process of intracellular membrane trafficking, vesicles are shuttled between the different compartments of a cell. Key proteins involved in vesicular transport are the SNARE (soluble NSF attachment protein receptor, where NSF stands for *N*-ethylmaleimide-sensitive factor) family of membrane proteins [13–15]. SNAREs are small transmembrane proteins that can be roughly classified into two broad categories: vesicle-SNARES (v-SNAREs, found on vesicles) and target-SNAREs (t-SNAREs, found on target compartments) [14,16]. The main function of SNAREs is to form cytoplasmic coiled-coil bundles to bridge vesicle and target membranes as they fuse [17–19]. Following membrane fusion, the ATPase NSF (*N*-ethylmaleimide-sensitive factor) and its adaptor protein α-SNAP (soluble NSF attachment protein) are required for the disassembly of SNARE complexes so that the SNAREs can be recycled [18]. In mammalian cells, NSF function is thus required for the maintenance of most SNARE-mediated membrane traffic.

Evidence is now emerging that SNARE-mediated membrane traffic is directly required for normal cell adhesion and migration. Previously, we have reported that inhibition of SNARE function impaired the trafficking of integrin $\alpha_5\beta_1$ and impeded the extension of lamellipodia as well as migration [20] and adhesion [21] in CHO-K1 cells. Recently, it has also been

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Abbreviations: ECM, extracellular matrix; CHO-K1, Chinese hamster ovary; FA, focal adhesion; FN, fibronectin; NSF, *N*-ethylmaleimide sensitive fusion protein; SNARE, soluble NSF attachment protein receptor; VAMP, vesicle-associated membrane protein

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found that the function of cellubrevin/VAMP3, an endosomal v-SNARE, is required for trafficking of β_1 integrins and migration in MDCK cells [22]. The latter study also demonstrated that inactivation of cellubrevin/VAMP3 altered cellular adhesion on ECM proteins.

In the present study, we characterize the cellular defect, specifically in cell spreading, that is caused by inhibition of SNARE function. We report that inhibition of SNARE-mediated trafficking pathways using a dominant-negative form of *N*-ethylmaleimide-sensitive factor (NSF) dramatically impairs the formation of lamellipodia that contributes to CHO-K1 cell spreading after attachment to fibronectin. Blocking SNARE function did not alter the number of FAs that formed in attached cells, but did lead to an increased number of larger FAs. The expression of constitutively active Rac1 partially restored spreading in cells lacking SNARE function but did not reduce the number of enlarged FAs in these cells. Interestingly, dominant-negative RhoA, which as expected reduced FA formation in CHO-K1 cells, had little effect on the formation of FAs in cells expressing dominant-negative NSF. By contrast, the effect that E329Q-NSF had upon FA formation was abrogated by treatment of cells with the ROCK inhibitor Y-27632. These results suggest that, during cell spreading, SNARE-mediated trafficking pathways contribute to RhoA-regulated FA formation.

2. Materials and methods

2.1. Reagents

The following mouse monoclonal antibodies were used: Anti-ILK, clone 65.1.9 (Upstate); Anti-Paxillin, clone 5H11 (Upstate), Anti-Vinculin (Sigma) and Anti-NSF (Stressgen). The goat polyclonal anti-fibronectin receptor (integrin $\alpha_5\beta_1$) was from Chemicon International. The following secondary antibodies were used: rabbit anti-mouse Alexa 594, goat anti-mouse Alexa 594, goat anti-rabbit Alexa 594, rabbit anti-goat Alexa 594. Secondary antibodies, rhodamine-conjugated phalloidin and DAPI were purchased from Molecular Probes. DAKO fluorescent mounting medium was obtained from DAKO Cytomation (Carpinteria, CA). FuGENE 6 transfection reagent was purchased from Roche. The ROCK inhibitor Y-27632 was purchased from Calbiochem. All other reagents were purchased from Sigma.

pcDNA3.1-wtNSF and pcDNA3.1-E329Q-NSF were described previously [23]. Constructs for expression of the catalytic light chain of Tetanus toxin, full-length SNAP23 and truncated SNAP23 were kind gifts of Dr. William Trimble (The Hospital for Sick Children, Toronto, Canada). Wild-type and mutant Rac1 and RhoA constructs were generous gifts of Dr. Katherine Siminovitch (Mt. Sinai Hospital, Toronto, Canada). cDNAs for paxillin, Rac1 (wild-type and mutant) and RhoA (wild-type and mutant) were cloned into pEGFP-C1 vector purchased from Clonetech Laboratories Inc. (Pablo Alto, CA).

2.2. Cell culture

Chinese hamster ovary (CHO-K1) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 7% fetal bovine serum (Sigma) under 5%CO_2 at 37 °C.

2.3. Cell transfections

Cells were transfected at 50–70% confluence with FuGENE 6 transfection reagent as described in the manufacture's protocol. Transfection efficiencies were routinely between 40% and 60%. In samples transfected with more than one expression vector, cells were co-transfected with pcDNA3.1, wild-type NSF, E329Q-NSF, Tetanus toxin light chain or SNAP23 construct together with soluble EGFP or EGFP-tagged paxillin in a 10:1 molar ratio, respectively. Replicate samples were stained for NSF to confirm that GFP-positive cells were also expressing NSF. All assays requiring NSF transfections lasted for a total of 8–10 h from the time of transfection until the time that the cells were fixed; those involving TeTx, 24 h; and SNAP23 constructs, 18 h.

2.4. Immunofluorescence labeling

Immunofluorescence staining was performed as described previously [20]. Cultured cells were first fixed with 4% paraformaldehyde for 15 min then washed with phosphate buffered saline (PBS). Following this, the cells were quenched with 0.15 M glycine/PBS and permeabilized with 0.2% Triton X-100. The fixed cells were blocked in 5% skim milk/PBS. Blocked samples were stained with primary antibody diluted in PBS for 1 h at 37 °C. After washing, the samples were incubated with secondary antibody (1:2000 dilution) or Rhodamine-conjugated phalloidin (1:5000 dilution) in 5% skim milk/PBS for 1 h at room temperature. Stained coverslips were washed with PBS and incubated with 300 nM DAPI/PBS to stain the nuclei. The coverslips were mounted on glass slides using DAKO fluorescent mounting medium.

2.5. Adhesion assays

Cells were detached using 5 mM EDTA/PBS and plated on glass coverslips that had been coated with fibronectin (20 μg/ml). The samples were incubated at 37 °C and 5% CO_2 for indicated times to allow attachment and/or spreading. Unattached cells were washed away with PBS and cells adherent to the coverslips were fixed and stained as described above. Control samples included populations of cells that had not been transfected or treated in any way or populations that had been transfected with expression vector (e.g., pcDNA3.1) alone.

2.6. Quantification of cell spreading and focal adhesion size

Specimens were imaged using a Leica DM-IRE2 inverted microscope with a Leica TCS SP2 scanning head (Leica, Heidelberg, Germany) and a 63× oil immersion lens. Images of GFP-paxillin and rhodamine-phalloidin were captured from cell samples. The ventral cell area (cellular footprint), number of FAs and FA size were quantified from confocal images of transfected cells that had been subjected to the adhesion assay as described above. Confocal images were captured at the ventral surface of the cells and then imported into ImageJ software (NIH, Bethesda, MD, USA) for analysis. Individual cells were manually outlined and their areas were determined. To assess the capacity of the cells to spread after attachment to the fibronectin substrate, the ventral area of cells beyond that obtained by cells treated with cytochalasin D was determined. This was done by subtracting the average ventral area (312 μm^2) that cells could achieve after spreading for 90 min when treated with 2 μM cytochalasin D. Increases in ventral cell area above this level were attributed to actin-driven formation of lamellipodia. Measurements of FA size and number were obtained in ImageJ using a minimum object size limit of 0.3 μm^2. The Student's *t*-test was used for statistical analysis of quantitative data.

2.7. RhoA activation assay

CHO-K1 cells stably expressing E329Q-NSF under the control of a tetracycline-inducible promoter (Clontech Laboratories, Mountain View, CA) have been described previously [20]. The cells were cultured in DMEM containing 10% FBS (tetracycline-free), hygromycin and G418 (BD Biosciences, Mississauga, Ont.) and were either left untreated or stimulated with 2 μg/mL doxycycline for 8 h to induce expression of mutant NSF. Cells were collected, plated on dishes coated with 10 μg/mL FN for the indicated time periods and then lysed in buffer containing 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, 10 mM sodium fluoride and protease inhibitor cocktail (Sigma, Oakville, Ont.). The lysates were precleared and equal amounts of protein for each time point were incubated with 60 μL of Rhotekin-RBD beads (Cytoskeleton, Denver, CO) for 60 min. The beads were collected by centrifugation and washed three times with lysis buffer. The pellets were re-suspended in SDS sample buffer, heated, and the eluted proteins were analyzed by SDS–PAGE and Western blotting. RhoA was detected using anti-Rho antibody (Cytoskeleton, Denver, CO) and secondary antibody coupled to horseradish peroxidase followed by ECL Plus chemiluminescence (Amersham Biosciences, Piscataway, NJ).

3. Results

3.1. Inhibition of NSF impairs cell spreading after attachment to FN

Recent studies suggest that SNARE function is required for cell adhesion and migration [20–22]. To define the role of SNARE function during cell adhesion, we have inhibited SNARE function using a general inhibitor of SNARE proteins. NSF is a central regulator of SNARE activity [24,25] and inhibition of NSF avoids the compensatory effects that can be induced by the inhibition of individual SNARE proteins with overlapping functions. Specifically, a dominant-negative form of NSF (E329Q-NSF) has been well-characterized and used effectively to characterize the role of SNARE-mediated traffic in phagocytosis, secretion in mast cells, store-operated Ca^{2+} channel function, cell adhesion and cell migration [20,21,23,26,27].

We used GFP-paxillin both as a marker of transfection (co-transfected with NSF constructs) and as a marker of focal adhesion formation in CHO-K1 cells as they spread on FN-coated surfaces. In co-transfected samples, we consistently observed that greater than 97.3 ± 0.99% (mean of six experiments ± S.E.M., 275 cells scored) of cells expressing GFP-paxillin were also expressing the co-transfected construct. To accurately quantify the role of SNARE-mediated traffic in cell spreading, assays were conducted to determine the change in ventral cell area that occurs after cells have attached to an ECM protein. Within 30 min of contacting FN, CHO-K1 cells have attached and attained a mean ventral surface area of approximately 300 μm^2 (Fig. 1A, E and I). This was true of untreated cells, cells transfected with EGFP or pcDNA3.1 alone, and cells transfected with either wild-type NSF (Fig. 1A and I) or E329Q-NSF (Fig. 1E and I). This ventral area is similar to the area observed in cells treated with 2 μM cytochalasin D (Fig. 1 I, white bar) during adhesion to FN for 90 min. We therefore considered increases in ventral cell area beyond this level to be the product of actin-dependent, lamellipodium-based cell spreading and measured these changes as described in Section 2. Once attached to FN (30 min after plating), CHO-K1 cells begin to elaborate

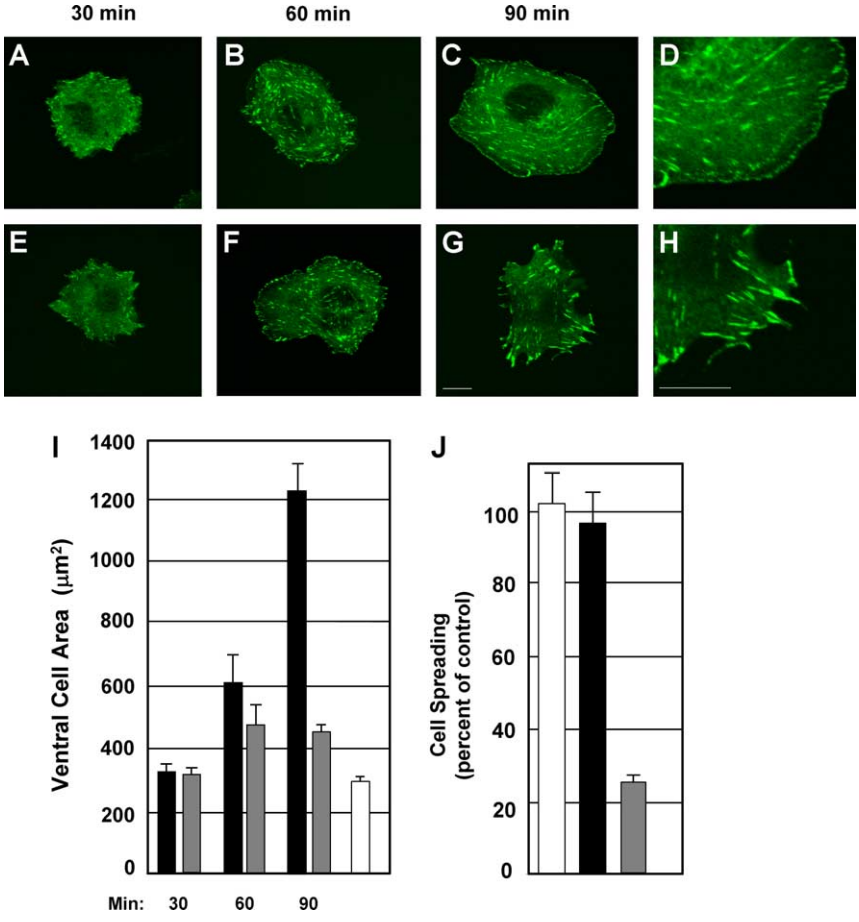

Fig. 1. Dominant-negative NSF impairs spreading in CHO-K1 cells. CHO-K1 cells were co-transfected with GFP-paxillin along with wild-type (wt-NSF) or dominant-negative NSF (E329Q-NSF). Cells were plated on FN and imaged after 30, 60 and 90 min. (A–D) Cells transfected with GFP-paxillin and wt-NSF. (E-H) Cells transfected with GFP-paxillin and E329Q- NSF. Panels D and H show enlarged view of cells in C and G, respectively. Scale bar = 10 μm. (I) 30, 60 and 90 min after plating on fibronectin, transfected cells were imaged and the ventral cell area was determined using ImageJ. Black bars, wt-NSF; grey bars, E329Q-NSF; white bar, non-transfected cells treated with 2 μM cytochalasin D and plated for 90 min. (J) The extent of cell spreading was calculated as the increase in ventral surface area between 30 and 90 min. The result is presented as percent of non-transfected control sample. White bar, pcDNA3.1 control; black bar, wt-NSF; grey bar, E329Q-NSF. In (I) and (J), more than 30 randomly selected cells were analyzed per sample in each experiment (>90 cells total per group). Means ± S.E.M. are shown from at least three independent experiments.

lamellipodia, spread out and form prominent paxillin-containing focal adhesions. This progression is readily observed 60 and 90 min after plating (Fig. 1), and we did not detect significant cell spreading beyond this in samples measured at later time points. The time-course of cell spreading was consistent between untreated cells (in which endogenous paxillin was monitored) and cells transfected with GFP-paxillin alone. Using GFP-paxillin as a marker of transfection, we observed that expression of E329Q-NSF impaired cell spreading by approximately 80% compared to controls (Fig. 1C, G, I, J). Wild-type NSF had no significant effect on cell spreading (Fig. 1J).

3.2. Dominant negative NSF increases the size of focal adhesions

The fact that dominant-negative NSF could block cell spreading without affecting cell attachment prompted us to examine the FAs in the inhibited cells to determine if they contained expected components of FAs, specifically $\alpha_5\beta_1$ integrin and vinculin. As seen in Fig. 2, inhibition of SNARE function with E329Q-NSF did not disturb the localization of $\alpha_5\beta_1$ integrin (Fig. 2E and F) or vinculin (Fig. 2G and H) in paxillin-containing structures.

Previously, we reported that blocking SNARE function did not alter the number of FAs that formed in the lamellipodia of migrating cells [20]. Consistent with these results, expression of dominant-negative NSF did not alter the number of focal adhesions formed per unit cell area as CHO-K1 cells adhere to FN (Fig. 2I). Close inspection of samples imaged in Figs. 1 and 2, however, did reveal that FAs in E329Q-NSF-expressing cells appeared to be larger than the FAs in control cells (see Fig. 1, panels D and H and Fig. 2, panels A, C, E and G). We therefore quantified the effect that blocking SNARE-mediated traffic had upon FA size in adherent cells. CHO-K1 cells were co-transfected with GFP-paxillin and either wild-type or E329Q-NSF and FA formation was monitored at 30, 60, 90 and 120 min. FAs were counted and measured using digital confocal micrographs and ImageJ software as described previously [20]. There were no significant differences in FA size between E329Q-NSF and wild-type NSF expressing cells at early time points of adhesion (within 60 min). At later time points,

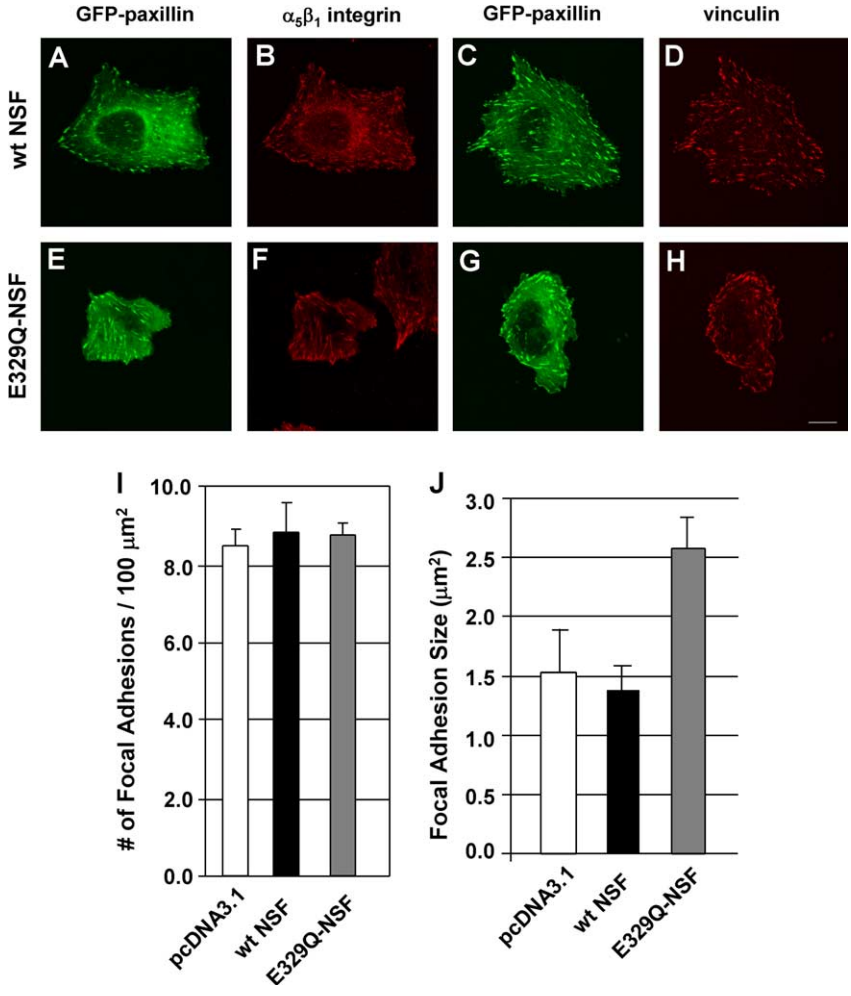

Fig. 2. Dominant-negative NSF alters focal adhesion size in CHO-K1 cells. CHO-K1 cells were co-transfected with GFP-paxillin along with wt NSF (A–D) or E329Q-NSF (E–H) for 6 h. Cells were then plated on fibronectin for 2 h, fixed and stained with antibodies to $\alpha_5\beta_1$ (A, B, E, F) or vinculin (C, D, G, H). Confocal images of the GFP-paxillin-containing structures in ventral sections of the cells were then captured. (A, C, E, G) GFP-paxillin; (B, F) $\alpha_5\beta_1$ integrin; (D, H) vinculin. Scale bar = 10 μm. (I, J) Confocal images of the GFP-containing structures in ventral sections of the cells were transferred into ImageJ and quantitatively analyzed. (I) Number of focal adhesions per 100 μm^2 ventral cell area. (J) Focal adhesion size (μm^2). White bar, pcDNA3.1 control; black bar, wt-NSF; grey bar, E329Q-NSF. More than 30 randomly selected cells were analyzed per sample in each experiment. Means ± S.E.M. of at least three independent experiments are shown.

control cells had typically sized FAs, consistent with FA sizes defined previously [2,28], while the FAs in cells transfected with E329Q-NSF appeared less homogeneous in size (see Figs. 1 and 2). Significantly, after 120 min of adhesion E329Q-NSF-expressing cells had a mean FA size 68% larger than that of control cells (Fig. 2J). The mean FA size was $2.55 \pm 0.29\ \mu m^2$ in E329Q-NSF-expressing cells and $1.52 \pm 0.44\ \mu m^2$ in control cells; $P < 0.05$. Similar results were obtained after 90 min of adhesion.

Expression of GFP-paxillin itself had a minor effect on FA structure, possibly through its function as a scaffolding protein. Nonetheless, we consistently observed increased FA size in cells expressing E329Q-NSF, whether we measured GFP-paxillin-containing FAs or endogenous paxillin by immunofluorescent staining. Together, these observations suggest that blocking SNARE-mediated membrane trafficking pathways with E329Q-NSF inhibits cell spreading and leads to the formation of larger FAs.

3.3. FAs in E329Q-NSF-expressing cells coincide with actin stress fibers

To determine if the FAs in cells expressing E329Q-NSF were interacting with the actin cytoskeleton, the colocalization of GFP-paxillin and F-actin structures was examined. The GFP-paxillin-containing FAs in both wt-NSF- and E329Q-NSF-expressing cells supported the insertion of actin stress fibers as indicated by co-localization of GFP-paxillin and rhodamine-phalloidin (Fig. 3). We could not discern any obvious differences in the number of GFP-paxillin-containing structures that contained F-actin between control, wt NSF-transfected or E329Q-NSF-transfected samples.

3.4. Expression of Tetanus toxin or truncated SNAP23 impairs cell spreading and alters FA formation

While the primary function of NSF is to regulate SNARE activity, it is has also been shown to form functional interactions with other intracellular targets [29,30]. To confirm the role of SNARE proteins in regulating FA formation, adhesion was examined in CHO-K1 cells transfected with the catalytic light chain of Tetanus toxin (TeTx) or with a truncated form of SNAP23. TeTx is a Clostridial protease that cleaves the SNAREs VAMP1, VAMP2 and VAMP3, of which only VAMP3 is expressed in CHO-K1 cells, and we have previously shown that expression of the catalytic chain of TeTx is an effective way to disrupt the function of VAMP3 in CHO-K1 cells [20]. Here, we report that TeTx expression impaired cell spreading (Fig. 4A) and had a small but reproducible effect on FA size (Fig. 4B).

SNAP23 is a plasma membrane SNARE found in most tissues [31,32]. As such, it is proposed to have an important function in SNARE-mediated membrane traffic, and exocytosis in particular, at the plasma membrane. To investigate SNAP23 function in cell adhesion, we transfected CHO-K1 cells with either full-length SNAP23 or a truncated form of SNAP23 ($SNAP23^{C\Delta 9}$). $SNAP23^{C\Delta 9}$ is lacking its 9 carboxyl-terminal amino acids and this truncated form is capable of forming non-functional complexes with SNARE partners and can therefore exert a dominant-negative effect on SNAP23 function. This mutant of $SNAP23^{C\Delta 9}$ has been shown to potently inhibit SNAP23-mediated secretion [33] and transferrin recycling [27]. We transfected CHO-K1 cells with $SNAP23^{C\Delta 9}$ and found that expression of this truncated form of SNAP23 markedly inhibited cell spreading (Fig. 4A). Furthermore, similar to the effect of expressing dominant-negative NSF, $SNAP23^{C\Delta 9}$ expression resulted in the formation of larger FAs as cells adhered to FN (Fig. 4B). Together, these results are consistent with previously published observations of SNARE function during cell adhesion [21,22] and suggest that the effects of impairing NSF function in the cells used here can be largely attributed to alterations in SNARE activity.

3.5. Constitutively active Rac1 does not restore focal adhesion size in E329Q-NSF-expressing cells

The GTPase Rac1 is known to regulate early steps in the process of integrin-mediated cell spreading [34,35]. It has been proposed that signaling through Rac1 can contribute to recruitment of high affinity integrins and lamellipodial extension [36]. We speculated that if Rac1 function is stimulated by signaling events emanating from integrins and promotes cell spreading, then constitutively active Rac1 might rescue spreading in cells expressing dominant-negative NSF and restore focal adhesion size. Cells were co-transfected with

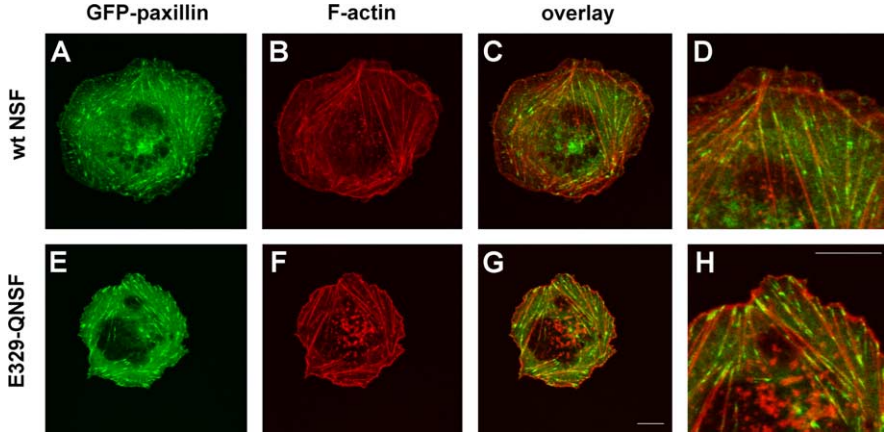

Fig. 3. FAs in E329Q-NSF-expressing cells are localized at the ends of actin stress fibers. CHO-K1 cells were co-transfected with GFP-paxillin along with either wt NSF (A–D) or E329Q-NSF (E–H) for 6 h and then plated on fibronectin for 2 h. Samples were fixed and stained with rhodamine-phalloidin and confocal images of GFP-paxillin-containing structures (A and E) and F-actin structures (B and F) in ventral sections of cells were captured. (C, G) overlays; (D, H) enlarged views of cells in C and G, respectively. Scale bars = 10 μm.

constitutively active Q61L-Rac1 together with either wild-type NSF or E329Q-NSF and spreading was analyzed as described above. Constitutively active Rac1 enhanced spreading in all cells in which it was expressed (Fig. 5) without altering cell surface integrin levels (data not shown). Cells expressing Q61L-Rac1 alone or wild-type NSF together with Q61L-Rac1 both exhibited a 60% increase in cell spreading (Fig. 5A). Cells co-expressing Q61L-Rac1 and E329Q-NSF exhibited more than a fourfold increase in spreading compared to cells expressing E329Q-NSF alone (Fig. 5A). Interestingly, cells expressing both E329Q-NSF and Q61L-Rac1 were unable to spread as extensively as cells expressing only Q61L-Rac1. Thus, the ability of CHO-K1 cells to spread in the presence of constitutively active Rac1 was dependent, in part, upon SNARE function. Importantly, constitutively active Rac1 had no effect on the increase in FA size that is induced by expression of E329Q-NSF (Fig. 5B). These results suggest that the impairment to cell spreading and the alteration to FA size that are caused by inhibition of SNARE-mediated membrane traffic are occurring downstream of Rac1 activity.

3.6. Dominant-negative NSF alters FA formation downstream of RhoA

During the course of integrin-mediated cell spreading, Rac1 signaling is required at an early stage, for the extension of lamellipodia, and subsequently RhoA actitvity leads to

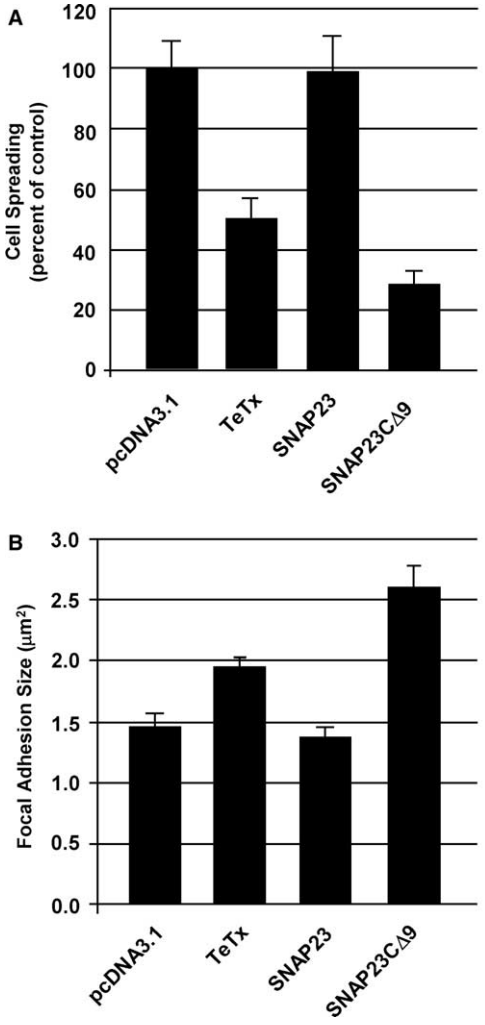

Fig. 4. The catalytic chain of Tetanus toxin and truncated SNAP23 impair cell spreading and alter FA formation. CHO-K1 cells were transfected with pcDNA3.1 vector alone, the catalytic light chain of Tetanus toxin (TeTx), full-length SNAP23 or truncated SNAP23 ($SNAP23^{C\Delta 9}$). (A) The extent of cell spreading was then calculated as the increase in ventral surface area that occurred between 30 and 90 min. The results are presented as percent of control sample. (B) Focal adhesion size (μm^2) was measured using confocal images of the GFP-containing structures in ventral sections of the cells and ImageJ software. In all experiments, more than 30 randomly selected cells were analyzed per sample. Means ± S.E.M. of at least three independent experiments are shown.

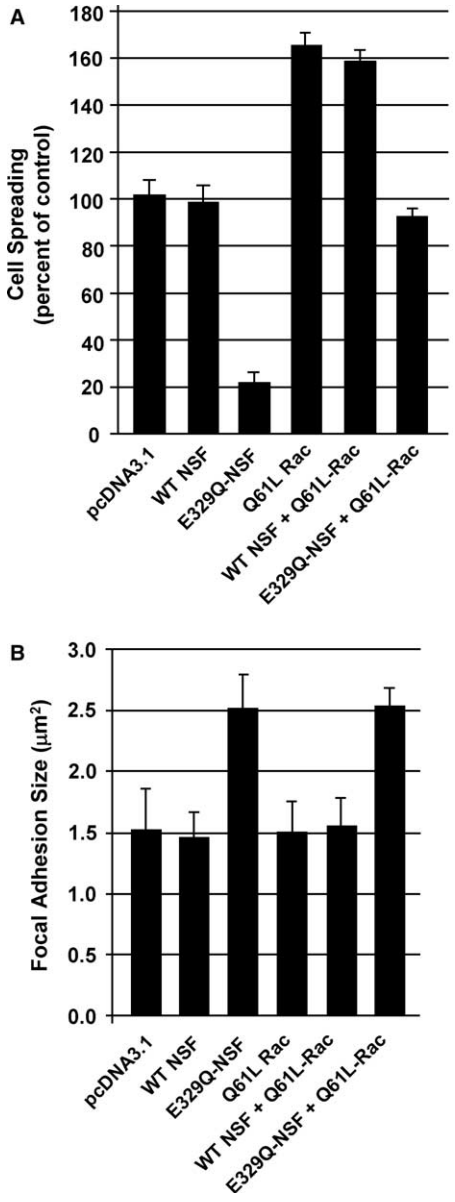

Fig. 5. Constitutively active Rac1 does not restore FA size in E329Q-NSF-expressing cells. CHO-K1 cells were transfected with GFP-Rac1-Q61L along with indicated constructs for 6–8 h, plated on fibronectin for 2 h and fixed. (A) Cell spreading: Cells were stained with rhodamine-phalloidin and antibodies to NSF and cell spreading was quantified as describe in Section 2. (B) Focal adhesion size: Samples were stained with antibodies to NSF and paxillin and FAs were measured as described previously. Means ± S.E.M. for at least three independent experiments are shown.

myosin-based contraction that drives integrins and signaling/scaffolding proteins into larger focal adhesions [3,37]. Given the tendency of E329Q-NSF-expressing cells to form larger FAs, even in cells in which Rac1 activity had been experimentally elevated, we postulated that E329Q-NSF-expressing cells have improperly regulated RhoA function, leading to increased FA size and altered cell spreading. We examined RhoA activity throughout the course of spreading in CHO-K1 cells and we did not detect significant differences in cellular levels of active RhoA between control cells or cells expressing E329Q-NSF (Fig. 6A). To directly assess RhoA function during cell adhesion and FA formation in CHO-K1 cells, these processes were analyzed in cells expressing a dominant-negative RhoA construct. Consistent with previous studies [38], CHO-K1 cells expressing GFP-tagged dominant-negative RhoA (T19N-RhoA-GFP), but not wild-type RhoA, displayed significantly reduced levels of spreading (Fig. 6B). This reduced spreading may be due to the fact that RhoA activity is required for normal FA formation and it is therefore possible that RhoA function and SNARE-mediated membrane traffic are influencing FA formation through a common mechanism. We examined the consequences of inhibiting RhoA function on the formation of FAs within cells in which SNARE function was impaired. T19N-RhoA-GFP was co-expressed with wt-NSF or E329Q-NSF in CHO-K1 cells. The cells were then examined for spreading and FA adhesion formation as described above. As expected, T19N-RhoA-GFP expression inhibited FA assembly in control cells (Fig. 6C and D). Significantly, T19N-RhoA-GFP did not inhibit FA formation in cells expressing E329Q-NSF (Fig. 6 E and F). Mean FA size was $2.49 \pm 0.23\ \mu m^2$ in cells transfected with E329Q-NSF alone, and $2.4 \pm 0.33\ \mu m^2$ in cells transfected with both

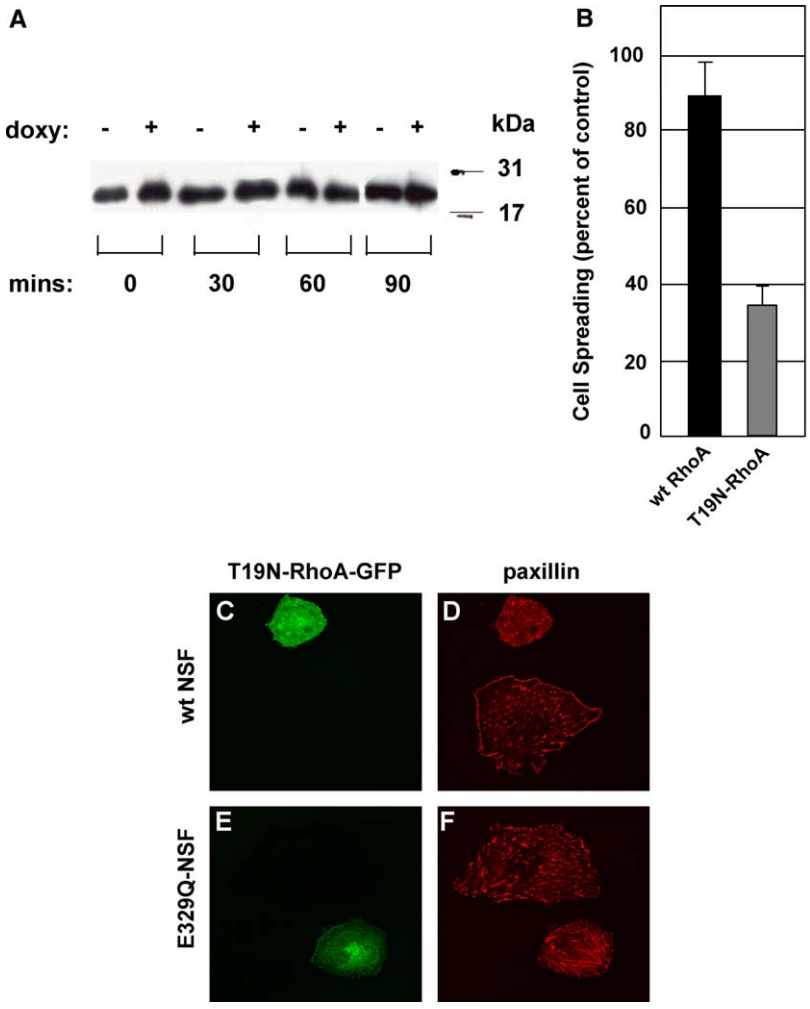

Fig. 6. E329Q-NSF alters FA size downstream of RhoA. (A) Detection of active RhoA in E329Q-NSF-expressing CHO-K1 cells. CHO-K1 cells containing E329Q-NSF under the control of a tetracycline-inducible promoter were treated with DMSO (−), as control, or 2 μg/mL doxycycline (+) to induce expression of E329Q-NSF. Cells were plated on FN for the indicated times and active RhoA was isolated from cell lysates using Rhotekin-RBD beads and detected by Western blotting with antibody against RhoA. (B) Dominant-negative RhoA impairs spreading in CHO-K1 cells. Cells were transfected with vector alone, wt RhoA-GFP or T19N-RhoA-GFP for 16 h and then plated on fibronectin for 2 h. The extent of cell spreading was calculated as increase in ventral cell area after 30 min of attachment. The result is presented as percent of vector-transfected control sample. Black bar, wt RhoA-GFP; grey bar, T19N-RhoA-GFP. Means ± S.E.M. of three independent experiments are shown. (C–F) Dominant-negative RhoA does not block focal adhesion formation in E329Q-NSF-expressing cells. Cells were co-transfected with T19N-RhoA-GFP along with wt NSF (C, D) or E329Q-NSF (E, F). The cells were plated on fibronectin for 2 h, fixed and stained with anti-paxillin antibody (D, F). C and E show T19N-RhoA-GFP; D and F show paxillin. Scale bar in $F = 10\ \mu m$.

E329Q-NSF and T19N-RhoA-GFP. These findings suggest that SNARE-mediated membrane traffic is involved in focal adhesion formation downstream of RhoA activity.

3.7. Formation of FAs in E329Q-NSF-expressing cells is blocked by inhibition of ROCK

RhoA activity is known to influence the structure of actin stress fibres and the maturation of FAs within spreading cells through its effector Rho kinase (ROCK) [39,40]. We examined the involvement of ROCK in FA formation in cells expressing E329Q-NSF by treating cells with the ROCK inhibitor Y-27632 (20 μM). In wild-type NSF-expressing cells and E329Q-NSF-expressing cells, inhibition of ROCK significantly reduced the formation of FAs (Fig. 7C and I). The effect of inhibiting ROCK was quite potent; the size of FAs in Y-27632-treated cells was reduced to below that which could be reliably quantified. In contrast to this, an inhibitor of myosin light chain kinase, ML-7 (5 μM), had no significant effect on focal adhesion size in E329Q-NSF-expressing cells (Fig. 7H). Consistent with previous studies [41], ML-7 did alter the formation of peripheral FAs; however, this effect was not influenced by E329Q-NSF expression. These observations suggest that the enlarged FAs resulting from the inhibition of SNARE-mediated traffic can be partially attributed to the activity of ROCK and its effectors.

4. Discussion

The present study demonstrates that SNARE-mediated trafficking pathways are required for normal focal adhesion (FA) formation during spreading in CHO-K1 cells. Specifically, we have found that inhibition of SNARE function by the expression of dominant-negative NSF (E329Q-NSF) impaired cell spreading but did not prevent FA formation. Importantly, it was observed that E329Q-NSF expression in cells caused the formation of larger FAs, relative to the FAs formed in control cells. Similar results were obtained when the function of a plasma membrane SNARE, SNAP23, was inhibited by expression of a truncated mutant of this SNARE. Close analysis of the FAs in E329Q-expressing cells indicated that they were structurally similar to those in control counterparts; the FAs were localized at the tips of F-actin stress fibers and contained expected FA proteins – $\alpha_5\beta_1$ integrin, paxillin and vinculin. While these observations suggest that, except for their increased size, the FAs in E329Q-NSF-expressing cells are nor-

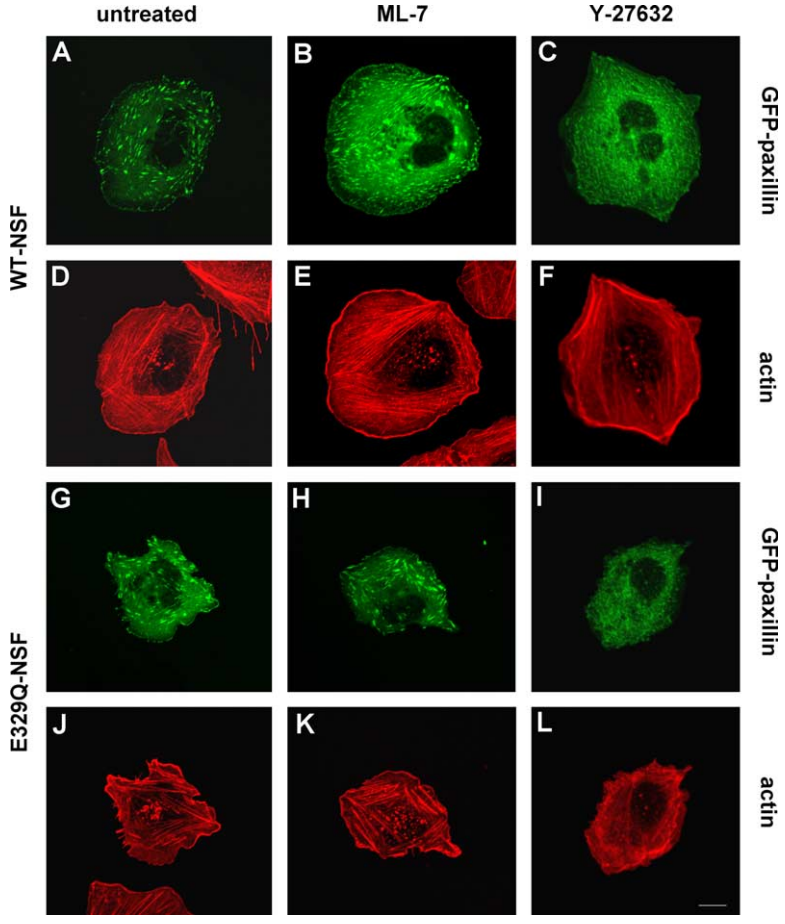

Fig. 7. Formation of FAs in E329Q-NSF-expressing cells is impaired by inhibition of ROCK. CHO-K1 cells were co-transfected with GFP-paxillin along with either wt-NSF (A–F) or E329Q-NSF (G–L) for 8 h and then plated on fibronectin for 2 h. (B, E, H, K) Samples treated with 5 μM ML-7. (C, F, I, L) Samples treated with 20 μM Y-27632. Inhibitors were added 1 h prior to plating and maintained throughout the course of spreading. Samples were then fixed and stained with rhodamine-phalloidin. GFP-paxillin (A–C, G–I) and F-actin (D–F and J–L) were imaged as described above. Scale bar in $L = 10$ μm.

mal, it remains possible that inhibiting SNARE function affected the distribution of FA proteins not examined here. It is also possible that the protein content of FAs has been altered quantitatively. We are currently investigating these possibilities.

Previously, we have shown that blocking SNARE function leads to the accumulation of $\alpha_5\beta_1$ in a Rab11-containing endosomal compartment and produces a modest decrease in cell surface $\alpha_5\beta_1$ integrin expression in CHO-K1 cells [20]. While it is possible that the decrease in cell surface integrin expression caused by E329Q-NSF could lead to impaired signaling downstream of integrin engagement, which could in turn affect FA size, it is not clear how decreased cell surface integrin would result in larger FAs. This hypothesis is also not consistent with the results of our experiments here using constitutively active Rac1. Rac1 is known to function downstream of integrins, and expression of Q61L-Rac1 in the CHO-K1 cells used here dramatically stimulated cell spreading. Significantly, Rac1-stimulated cell spreading occurred without altering cell surface integrin levels and without restoring FA size in cells expressing dominant-negative NSF. From these findings, it is concluded that during cell adhesion Rac1 signaling impinges upon more than one biochemical pathway, including pathways that control the formation of lamellipodia and FAs. These Rac1-regulated pathways appear to be connected, but partially independent. Thus, it is plausible that Rac1 activity can contribute to lamellipodium extension in the absence of SNARE function, through the stimulation of actin-driven membrane protrusion, but under such conditions spreading is not optimal because FA formation is not normal.

The preceding results suggest that SNARE-mediated membrane traffic may be required for the delivery of factors that regulate FA formation and maturation at sites of active cell spreading. Candidate factors in this process include signaling molecules involved in regulating FA assembly and/or disassembly. For example, Rac has been shown to regulate the selective recruitment of high-affinity integrins to sites of lamellipodium formation in endothelial cells [36]. In turn, it has been suggested that these integrins induce localization of Rac-GTP to the cell edge allowing effector binding [42]. One such effector, PAK, has been shown to promote cell spreading and inhibit actomyosin-based formation of FAs [43]. If PAK were not properly localized to sites of cell spreading, a decrease in spreading and alterations to FA morphology could result, consistent with our observations.

The finding that SNARE function regulates FA formation downstream of Rac1 raised the possibility that SNARE-mediated traffic is required to limit RhoA function during FA formation. Much evidence supports the notion that during cell spreading RhoA functions subsequently to Rac1, inducing actin stress fiber formation and stimulating contractile forces that generate FAs. During the first 10–20 min of integrin-mediated cell adhesion, levels of RhoA activity have been found to be relatively low [1,44–46]. It has been proposed that this reduced RhoA activity limits actinomyosin contraction and promotes Rac-regulated lamellipodium formation [47,48]. The observation that E329Q NSF-expressing cells have enlarged FAs suggested that RhoA activity is not properly regulated in these cells, leading to excessive RhoA-induced contractility, enlarged FAs and reduced spreading. We report no dramatic differences in RhoA activity as a result of inhibiting SNARE function in CHO-K1 cells; however, our results do indicate that RhoA function is required for spreading in these cells. Interestingly, dominant-negative RhoA (T19N-RhoA) inhibited both spreading and FA assembly in control cells, as expected, but did not significantly alter FA formation in cells expressing dominant-negative NSF. By contrast, the ROCK inhibitor Y-27632 abrogated the effect that inhibition of SNARE function had on FA formation. These observations thus suggest that blocking SNARE-dependent traffic alters FA formation downstream of RhoA activation, and in a manner that is dependent on ROCK function.

Collectively, our data are consistent with a model of cellular adhesion in which spreading is supported by SNARE-mediated membrane traffic that facilitates modulation of RhoA-regulated FA formation. In this context, SNARE function may mediate the delivery of regulators of stress fiber formation, helping to modulate contractility so that FAs form while contraction is balanced with membrane protrusion. We postulate that, during cell spreading, SNARE-mediated traffic delivers components that support membrane extension as well as factors that modulate actin-based contractility, for example regulators of myosin light chain phosphatase. In this way the forces of membrane protrusion and actinomyosin contraction may be properly balanced, promoting lamellipodium extension and limiting FA size. When SNARE function is blocked, necessary regulatory factors may be mis-localized, ROCK-regulated contraction may not be properly attenuated and, as observed here, decreased spreading may be accompanied by increased FA size.

Together with work recently published by our laboratory and others, the present findings clearly indicate a role for SNARE-mediated membrane traffic in cellular adhesion. We can now further conclude that SNARE function is required for the regulation of FA formation (and/or maturation) and signaling events downstream of the small GTPases Rac1 and RhoA. Furthermore, the biochemical events that contribute to FA size, and that are modulated by SNARE-mediated traffic, are dependent on the RhoA effector ROCK. Future studies are aimed at providing additional insight into the role of SNARE-mediated membrane traffic during cell adhesion, helping to elucidate the molecular mechanisms behind this important form of cellular motility.

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