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SNARE-mediated trafficking of $\alpha_5\beta_1$ integrin is required for spreading in CHO cells ☆

Michael Skalski, Marc G. Coppolino *

Department of Molecular and Cellular Biology, University of Guleph, Guelph, Ont., Canada N1G 2W1

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Abstract

In this study, the role of SNARE-mediated membrane traffic in regulating integrin localization was examined and the requirement for SNARE function in cellular spreading was quantitatively assessed. Membrane traffic was inhibited with the VAMP-specific catalytic light chain from tetanus toxin (TeTx-LC), a dominant-negative form (E329Q) of *N*-ethylmaleimide-sensitive fusion protein (NSF), and brefeldin A (BfA). Inhibition of membrane traffic with either E329Q-NSF or TeTx-LC, but not BfA, significantly inhibited spreading of CHO cells on fibronectin. Spreading was rescued in TeTx-LC-expressing cells by co-transfection with a TeTx-resistant cellubrevin/VAMP3. E329Q-NSF, a general inhibitor of SNARE function, was a more potent inhibitor of cell spreading than TeTx-LC, suggesting that tetanus toxin-insensitive SNAREs contribute to adhesion. It was found that E329Q-NSF prevented trafficking of $\alpha_5\beta_1$ integrins from a central Rab11-containing compartment to sites of protrusion during cell adhesion, while TeTx-LC delayed this trafficking. These results are consistent with a model of cellular adhesion that implicates SNARE function as an important component of integrin trafficking during the process of cell spreading.

Keywords: Adhesion; Integrin; Membrane traffic; NSF; VAMP3

The process of integrin-mediated cell adhesion requires the precise regulation of integrin localization and activity, reorganization of the actin cytoskeleton, and localized membrane remodeling. Engagement of ECM proteins via cell surface integrin receptors causes a conformational change in and clustering of the integrins, which in turn triggers the association of signaling proteins, such as focal adhesion kinase (FAK), and scaffolding proteins, such as paxillin, vinculin, α-actinin, and talin, at the cytoplasmic domains of integrin subunits [1,2]. The association of signaling and scaffolding proteins with integrins, in "focal complexes," leads to activation of signaling pathways that induce the actin-propelled formation of Rac1-dependent lamellipodia and/or Cdc42-dependent filipodia. As the cell spreads, a shift in signaling occurs away from Rac1/Cdc42 signaling, leading to heightened RhoA signaling; as this occurs, integrins and scaffold proteins in focal complexes are forced into larger adhesive structures (focal adhesions) via myosin-based contraction. Spreading stops once a balance is achieved between actin-driven edge propulsion and forces generated by both myosin contraction and membrane tension [3].

☆ *Abbreviations:* Arf, ADP-ribosylation factor; BfA, brefeldin A; NSF, *N*-ethylmaleimide-sensitive fusion protein; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; EGF, epidermal growth factor; FAK, focal adhesion kinase; FBS, fetal bovine serum; FN, fibronectin; GEF, guanine nucleotide exchange factor; GFP, green fluorescent protein; PDGF, platelet-derived growth factor; SNARE, soluble NSF attachment protein receptor; TMR-Tfn, tetramethylrhodamine-labeled transferrin; TeTx-LC, tetanus toxin light chain; VAMP, vesicle associated membrane protein.

* Corresponding author. Fax: +1 519 837 1802.
E-mail address: mcoppoli@uoguelph.ca (M.G. Coppolino).

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Integrin-mediated cell spreading is dependent on the deployment of integrins to the cell surface, particularly at sites where new cell–ECM contacts are to be formed. Several recent studies have determined that intracellular trafficking of integrins is required for spreading and migration. These studies have shown a requirement for Arf6 [4], Rab4 [5,6], Rab11 [5], Rab5 [7], Rab8 [8], and dynamin [9] GTPases in cell motility. These GTPases are part of multi-step membrane trafficking pathways, where Arf-family proteins and dynamin control budding at donor membranes, and Rab GTPases and their effectors control tethering and docking at target membranes [10,11]. It is via a series of steps regulated in this manner that integrins have been shown to traffic through an endocytic-recycling pathway, from the early endosome to the recycling endosome and then to the cell surface. For example, EGF- and PMA-induced cell spreading has been found to involve exocytosis of β_1 integrins from the recycling endosome to the leading edge in a pathway dependent on Arf6 and Rab11 [12]. Integrins have also been observed to traffic from early endosomes directly to the plasma membrane via a Rab4-dependent pathway. This pathway was found to enhance adhesion and spreading, but this 'rapid-cycling' was specific for $\alpha_v\beta_3$ integrin under PDGF stimulation; $\alpha_5\beta_1$ integrin, and $\alpha_v\beta_3$ in non-stimulated cells, continued to traffic through the recycling endosome in a Rab11-dependent way [5]. Both studies suggest that recycling endosomes may contain a reservoir of integrins for use in attachment at the leading edge during spreading and/or migration.

In the trafficking of integrin receptors during adhesion, how the final stages of the pathways are regulated is not well understood. Recent studies from our laboratory [13] and other's [14] have implicated the function of SNARE proteins in cell adhesion and migration. Most known membrane traffic pathways culminate in SNARE (soluble NSF attachment protein receptor)-regulated membrane fusion events. Specifically, docking and membrane fusion are regulated by membrane bound SNAREs found on the vesicle (v-SNARE) and target (t-SNARE) membranes [15]. SNARE-mediated traffic is thus potentially involved in several steps during integrin trafficking, including steps that connect early endosomes with recycling endosomes and steps that target integrins to the plasma membrane. Although these trafficking pathways are likely to have significant effects upon cell–ECM interactions, the SNARE-mediated pathways that control integrin traffic during cellular spreading remain to be fully characterized.

In the present study, we quantitatively assessed the requirement for SNARE-mediated membrane traffic during integrin-mediated spreading in CHO cells and investigated the mechanism by which SNAREs contribute to this process. We report that inhibition of the v-SNARE cellubrevin/VAMP3, a known component of recycling endosomes, by expression of the catalytic light chain of tetanus toxin (TeTx-LC) reduced the extent of $\alpha_5\beta_1$-mediated cell spreading on fibronectin (FN). Cell adhesion was more potently impaired by expression of dominant negative NSF (E329Q-NSF), which acts as a general inhibitor of SNARE-mediated membrane traffic. Neither of these disruptions to SNARE function altered cell attachment to FN. Significantly, blocking SNARE function with either TeTx-LC or E329Q-NSF impaired the transport of $\alpha_5\beta_1$ integrin from a central Rab11-containing compartment to sites of lamellipodium protrusion during cell spreading. These results define the role of SNARE-mediated membrane traffic in the movement of integrins through endosomal recycling compartments, a process which is required for normal cellular adhesion.

Materials and methods

Materials. All chemicals were purchased from Sigma Chemical (St. Louis, MO) unless indicated otherwise. Goat polyclonal antibody against TeTx was obtained from Biodesign International (Saco, ME) and the mouse anti-NSF antibodies were purchased from Stressgen (San Diego, CA). The goat anti-FN receptor ($\alpha_5\beta_1$ integrin) and rabbit anti-NSF antibodies were from Chemicon (Temecula, CA). Antibodies against Rab11 were obtained from BD Biosciences (Mississauga, Ont.). Rabbit polyclonal anti-mannosidase II antibody was a kind gift from Dr. M. Farquhar (University of California at San Diego, CA). All secondary antibodies and rhodamine-labeled phalloidin were purchased from Invitrogen (Mississauga, Ont.).

The generation of the pcDNA3.1-wtNSF and pcDNA3.1-E329Q-NSF plasmids and their characterization are described elsewhere [16]. The pcDNA3.1-TeTx-LC, peGFP-VAMP3, and peGFP-vwVAMP3 were kind gifts from Dr. W.S. Trimble (Hospital for Sick Children, Toronto).

Cell culture and transfection. Chinese hamster ovary (CHO)-K1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (Sigma, Oakville, Ont.), or 5% FBS and 5% FetalClone II (VWR International, Mississauga) at 37 °C and 5% CO_2. The CHO-K1 cells stably expressing E329Q-NSF are described elsewhere [13] and were cultured in DMEM with 10% tetracycline-free FBS, hygromycin and G418 (BD Biosciences, Mississauga, Ont.). Cells were transfected with the various plasmid constructs using either FuGene6 Transfection Reagent (Roche Applied Science, Laval, QC) following the manufacturer's suggested protocol or with $CaPO_4$:DNA complex co-incubation. DNA complexes were incubated on the cells for 10 h when transfecting with NSF constructs or 24 h when transfecting with TeTx-LC. In experiments using BfA, cells were pretreated for 3 h with 5 μg/mL BfA and BfA was maintained at this concentration throughout the experiments.

Cell attachment and spreading assays. All attachment and spreading assays were done on glass coverslips coated with FN. To coat, individual coverslips were first cleansed with 0.1 M NaOH, washed with PBS, and then incubated in 20 μg/mL FN in PBS for 3 h. Before use, the coverslips were washed with PBS to remove excess FN. Treated cells were placed onto coated coverslips for 0.5 h (attachment assays) or 1.5 h (spreading assays). After the allotted time, the coverslips were gently washed and the samples were fixed with 4% paraformaldehyde in PBS. Attachment was assessed by taking digitized images using a 10× lens and counting attached cells in washed and unwashed samples. At least 50 transfected cells were imaged per condition for each experiment. Spreading was quantified by capturing digitized images

using a 40× lens. The images were analyzed by outlining cells and measuring ventral cell area in ImageJ software. Increases in ventral cell area after 30 min attachment were considered as cell spreading. Statistical comparisons between treatment groups were performed using Student's *t* test.

Endocytic trafficking assays. For endocytosis assays, CHO-K1 cells plated on coverslips were transfected for 9 h with NSF constructs and serum-starved in DMEM for one hour before beginning the assay. The coverslips were washed with ice-cold PBS to remove any unlabeled transferrin. Tetramethylrhodamine-labeled transferrin (30 μg/mL) in pre-warmed, serum-free DMEM was applied to the coverslips and incubated for 10 min. The coverslips were washed with PBS, treated with acid wash (3% acetic acid, 0.5 M NaCl, pH 4.0) to remove surface bound tetramethylrhodamine-labeled transferrin (TMR-Tfn), washed with PBS again, and fixed with 4% paraformaldehyde. For exocytosis assays, CHO-K1 cells were serum-starved for 1 h in DMEM and loaded with 30 μg/mL TMR-Tfn in DMEM for 3 h. After loading, the surface bound Tfn was removed by acid wash and the cells were incubated for the indicated times to allow exocytosis of the internalized TMR-Tfn, after which the cells were fixed and stained as outlined below.

For integrin exocytosis assays, a modification of the protocol used by Powelka et al. [12] was applied. Briefly, cells were serum starved in DMEM for one hour, followed by incubation of $\alpha_5\beta_1$ integrin antibody (AB1950 'fibronectin receptor,' Chemicon, Temecula, CA) diluted 1:100 in DMEM for 3 h, to allow endocytosis of antibody-labeled integrins. After incubation with the antibody, samples were washed with ice-cold PBS and lifted with trypsin–EDTA, which removed surface bound antibody. The cells were plated on FN-coated coverslips in serum-free DMEM, incubated at 37 °C for 20, 40 or 60 min, and then fixed and stained as outlined below.

Immunofluorescence microscopy. After fixation, cell samples were quenched with 0.1 M glycine/PBS and permeabilized with 0.1% Triton X-100/PBS. Coverslips were washed with PBS, blocked with 5% skim milk powder in PBS, and then stained with primary antibody. All primary antibodies were diluted 1:100, except the TeTx antibody (1:75). Secondary antibodies and/or rhodamine-labeled phalloidin were diluted 1:2000 in blocking solution. Following incubation in secondary antibody, coverslips were washed with PBS, incubated with 300 nM DAPI (Invitrogen, Mississauga, Ont.) for 10 min to stain nuclei, washed again, and mounted. Samples were imaged using a 63× lens on a Leica DM-IRE2 inverted microscope with a Leica TCS SP2 scanning head (Leica, Heidelberg, Germany).

Flow cytometry. To asses cell surface integrin expression, all manipulations were done at 4 °C. Cells were lifted from culture plates using EDTA-PBS and resuspended in ice-cold EDTA-PBS supplemented with 0.5% BSA, penicillin, and streptomycin (wash buffer). Cells were pelleted by centrifugation at 400*g* for 2 min, resuspended in 500 μL of anti-$\alpha_5\beta_1$ antibody (Chemicon) diluted 1:100 in wash buffer, and incubated for 1–1.5 h rotating at 4 °C. After primary antibody staining, the cells were washed and incubated in secondary antibody for 1–1.5 h. The cells were washed and analyzed using a Coulter flow cytometer. Forward and side-scatter gates were positioned so as to include only single, whole cells. A minimum of 5000 positive cells were counted for each experiment.

Western blotting. Cells transfected for the appropriate times or treated with doxacycline were lifted with EDTA-PBS and lysed in PBS containing 1% Triton X-100, 0.1% SDS, and 0.5% deoxycholic acid. The lysates were cleared by centrifuging at 13,000 rpm for 10 min and 25 μg of protein was separated by 10% SDS–PAGE gel. The protein was transferred to PVDF membrane, which was then blocked in 5% skim milk powder/Tris-buffered saline with 0.1% Tween 20 (TBST). The membranes were probed with primary antibody diluted in TBST for 1.5 h, followed by HRP-conjugated secondary antibody in TBST for 1 h. Protein bands were detected by ECL Plus chemiluminescence (Amersham Biosciences, Piscataway, NJ).

Results

Expression of TeTx-LC inhibits cell spreading

Cellubrevin/VAMP3 is a well-characterized component of endocytic recycling pathways [17]. To examine the function of this SNARE, and the pathways it regulates during cell adhesion, we expressed the catalytic light chain of tetanus toxin (TeTx-LC) in CHO cells. TeTx-LC cleaves the vesicle SNAREs VAMP1, VAMP2, and VAMP3, of which only VAMP3 is expressed in the CHO cells used here [13]. Previous research has shown that cleavage of VAMP3 by TeTx-LC inhibits an exocytic pathway, resulting in the accumulation of transferrin within a recycling endosome in this cell type [18]. Similarly, we have recently shown that TeTx-LC inhibition of VAMP3 can impair the serum-stimulated mobilization of $\alpha_5\beta_1$ integrin out of a Rab11-containing compartment [13]. Herein, we used TeTx-LC to characterize the function of VAMP3 during CHO cell adhesion to FN; specifically, cell attachment and cell spreading were quantified separately. Expression of TeTx-LC did not alter the efficiency with which cells attached to FN (20 μg/mL) within 30 min of plating (Figs. 1A, B, and E). Cells expressing TeTx-LC did, however, show significantly diminished spreading (538.5 ± 48.3 μm^2 compared to 812.0 ± 66.0 μm^2 for untreated, $n = 5$; $p \leqslant 0.05$) (Figs. 1C, D, and F). For cell spreading, increases in ventral cell area after 30 min attachment to FN were calculated. The ventral cell area after 30 min attachment was typically 300 μm^2 (Figs. 1A and B) and this was also the maximal ventral cell area achieved by cells treated with cytochalasin D (313.3 ± 18.2 μm^2, $n = 4$). Thus, increases in cell area beyond this were considered to be due to lamellipodium-based spreading.

The effects of TeTx-LC on cell spreading suggested that a VAMP3-dependent recycling pathway is involved in spreading of CHO cells. The requirement for VAMP3 function in cell spreading was confirmed by co-transfecting TeTx-LC with a TeTx-resistant mutant form of VAMP3 tagged with GFP (vwV3-GFP). In this construct, Gln^{63} and Phe^{64} of VAMP3 are mutated to Val and Trp, respectively, producing a form of VAMP3 that is resistant to proteolysis by TeTx-LC [13,19]. As shown in Fig. 1F, co-expression of TeTx-resistant vwV3-GFP with TeTx-LC returned spreading to control levels (750.8 ± 11.8 μm^2, $n = 3$; $p \leqslant 0.05$ vs. TeTx-LC), while co-expression of GFP-tagged wild-type VAMP3 protein and TeTx-LC had no significant effect (654.2 ± 23.2 μm^2, $n = 3$; data not shown).

TeTx-LC expression alters the intracellular trafficking of $\alpha_5\beta_1$ integrin during adhesion

The observation that a VAMP3-dependent pathway is required for cellular spreading, but not attachment,

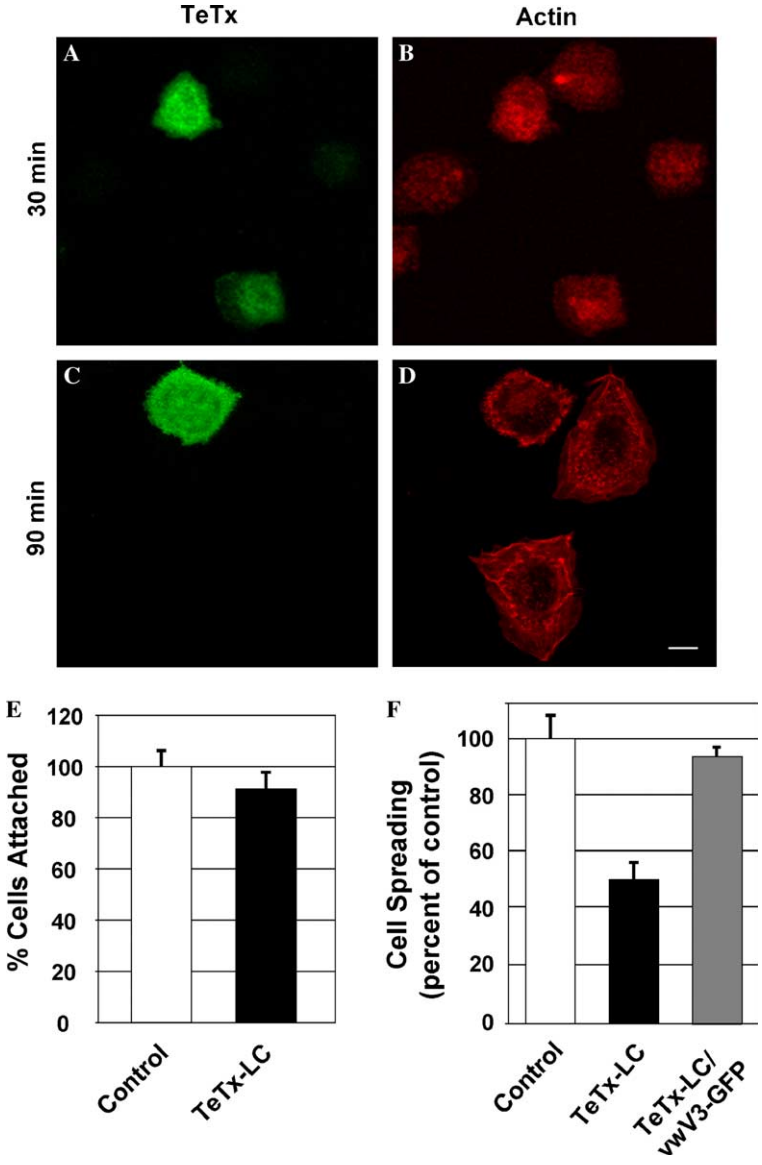

Fig. 1. TeTx-LC inhibits cellular spreading but not attachment to FN. CHO-K1 cells were transiently transfected with TeTx-LC for 24 h. The cells were then plated onto FN-coated (20 μg/mL) coverslips in serum-free medium. After 30 min (A,B) or 90 min (C,D), unattached cells were washed away and adherent cells were fixed and stained with anti-TeTx antibody (A,C) and rhodamine-phalloidin (B,D). Scale bar = 10 μm. (E) At 30 min, the percentage of attached transfected cells was determined (relative to unwashed controls). (F) At 90 min, the increase in ventral area of cells after attachment was quantified from digital photomicrographs using ImageJ software. (E,F) The results of at least three independent experiments are shown (means ± SEM), with at least 50 cells counted per experiment.

suggested that for normal spreading to occur membrane trafficking is required at a point after initial interaction with the ECM substrate. This could be explained by a lack of integrin receptors present on the cell surface, leading to a decrease in the efficiency with which membrane extensions (lamellipodia) attach to the substrate during spreading. Since $\alpha_5\beta_1$ is the primary integrin responsible for binding FN in CHO cells [20,21], it was determined if inhibition of VAMP3 function alters the level of $\alpha_5\beta_1$ integrin expression in these cells. CHO cells transfected with TeTx-LC or vector alone were analyzed for expression of $\alpha_5\beta_1$ integrin by Western blot. No differences in the levels of cellular $\alpha_5\beta_1$ integrin were detected as a result of TeTx-LC expression (Fig. 2A). Cells transiently co-transfected with GFP and either empty vector or TeTx-LC were also stained for cell surface $\alpha_5\beta_1$ integrin and quantified by flow cytometry. Apparent cell volume, as assessed by forward and side-scatter analysis, did not change between groups (data not shown). TeTx-LC expression did decrease cell surface $\alpha_5\beta_1$ integrin expression by approximately 17% compared to controls (TeTx-LC—82.3 ± 3.7% vs. empty vector; $n = 4$, $p \leqslant 0.05$) (Fig. 2B), as reported earlier [13]. Thus, blocking VAMP3 function decreased cell surface $\alpha_5\beta_1$ and impeded cell spreading on, but not attachment to, FN. Furthermore, since cell surface $\alpha_5\beta_1$ levels

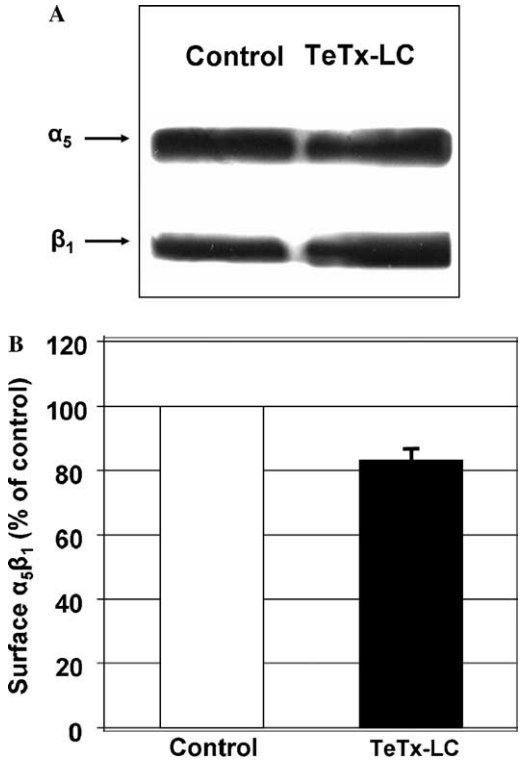

Fig. 2. TeTx-LC decreases surface expression of $\alpha_5\beta_1$ integrin. (A) Western blot for $\alpha_5\beta_1$ protein (using polyclonal anti-$\alpha_5\beta_1$ antibody) in control CHO-K1 cells and cells transfected with TeTx-LC for 24 h. (B) CHO-K1 cells were transiently co-transfected with 1:10 molar ratio of pEGFP and either pcDNA3.1-TeTx-LC (TeTx-LC) or empty vector (control) for 24 h. The cells were then harvested at 4 °C and stained for surface $\alpha_5\beta_1$ integrin expression using a polyclonal primary antibody to $\alpha_5\beta_1$ and a fluorescently labeled secondary antibody. Fluorescence of GFP-positive cells was quantified by flow cytometry. Forward and side scatter were also monitored to ensure cell volume did not change. At least 5000 cells were assessed per experiment, $n = 4$; mean ± SEM shown.

were decreased without alteration to total cellular $\alpha_5\beta_1$ content it was concluded that TeTx-LC was altering the intracellular compartmentalization of integrins during spreading.

To monitor the intracellular trafficking of $\alpha_5\beta_1$, integrin recycling assays were used. Cells transiently co-transfected with GFP and either vector alone or TeTx-LC were serum starved for 1 h. $\alpha_5\beta_1$ integrin was then labeled with antibody and allowed to be internalized as described in Materials and methods. As published previously by other laboratories [5,12], internalized $\alpha_5\beta_1$ was partially localized in a central, Rab11-containing compartment (Figs. 3A–C). Within 20 min of plating cells on FN, a significant amount of the labeled integrin had trafficked from the Rab11-positive compartment to the cell periphery (Figs. 3A and D). At this time-point, TeTx-LC impeded the redistribution of $\alpha_5\beta_1$ integrin from the central compartment to the periphery (compare Figs. 3D and E). Incubation for longer time periods revealed that $\alpha_5\beta_1$ was eventually mobilized out of the central Rab11-containing compartment in TeTx-LC-expressing cells (Fig. 3G). These results imply that a VAMP3-dependent trafficking pathway is required for efficient movement of $\alpha_5\beta_1$ integrin, from a prominent Rab11-containing compartment to the cell periphery, in support of normal spreading. The data also suggest that a VAMP3-independent pathway may compensate for lack of VAMP3 function, allowing for the delayed mobilization of $\alpha_5\beta_1$.

Brefeldin A does not inhibit cell spreading

The demonstration that blocking a VAMP3-dependent membrane trafficking pathway delays the redistribution of $\alpha_5\beta_1$ integrin suggests that additional membrane trafficking pathways might be involved in this process. The fungal metabolite brefeldin A (BfA) was used to characterize the possible contribution to cell spreading of the secretory pathway. Brefeldin A, which targets Arf1-specific GEFs, is known to inhibit retrograde trafficking within the Golgi apparatus as well as that from the Golgi to the ER, eventually leading to an inhibition of anterograde traffic [22,23]. In CHO cells, disruption of the Golgi after 3 h BfA treatment was confirmed by staining cells for the Golgi marker mannosidase II (Figs. 4A–D). Interestingly, CHO cells pretreated for 3 h with BfA showed no significant difference in spreading compared to untreated cells (Fig. 4E), yet the treated cells showed a similar drop in surface integrin expression to that of TeTx-LC-expressing cells (BfA—76.9 ± 3.6% vs. untreated; $n = 3$) (Fig. 4F). These findings suggest that the biosynthetic-secretory pathway, although contributing to the total surface pool of integrin, is not required for spreading of CHO cells.

Dominant-negative NSF impairs cell spreading

Our results indicate that VAMP3 function is required for normal spreading and efficient integrin trafficking in CHO cells. In contrast to this, BfA-sensitive traffic contributes to cell surface integrin expression, but not cell spreading. To fully define the extent to which SNARE-mediated membrane trafficking is involved in cell adhesion, the functions of many SNARE proteins will need to be examined. While a systematic analysis of all candidate SNAREs in this regard is underway, we herein sought to assess the role of SNARE-mediated membrane traffic in cell adhesion through the use of a general inhibitor of SNARE function. NSF is a central regulator of SNARE activity and has been shown to be required for most eukaryotic intracellular membrane trafficking pathways, including endocytosis, constitutive and regulated exocytosis, traffic between endosomes, and intra-Golgi traffic [24]. Inhibition of NSF with a dominant-negative form of NSF (E329Q-NSF) has been previously used to characterize the role of SNARE-med-

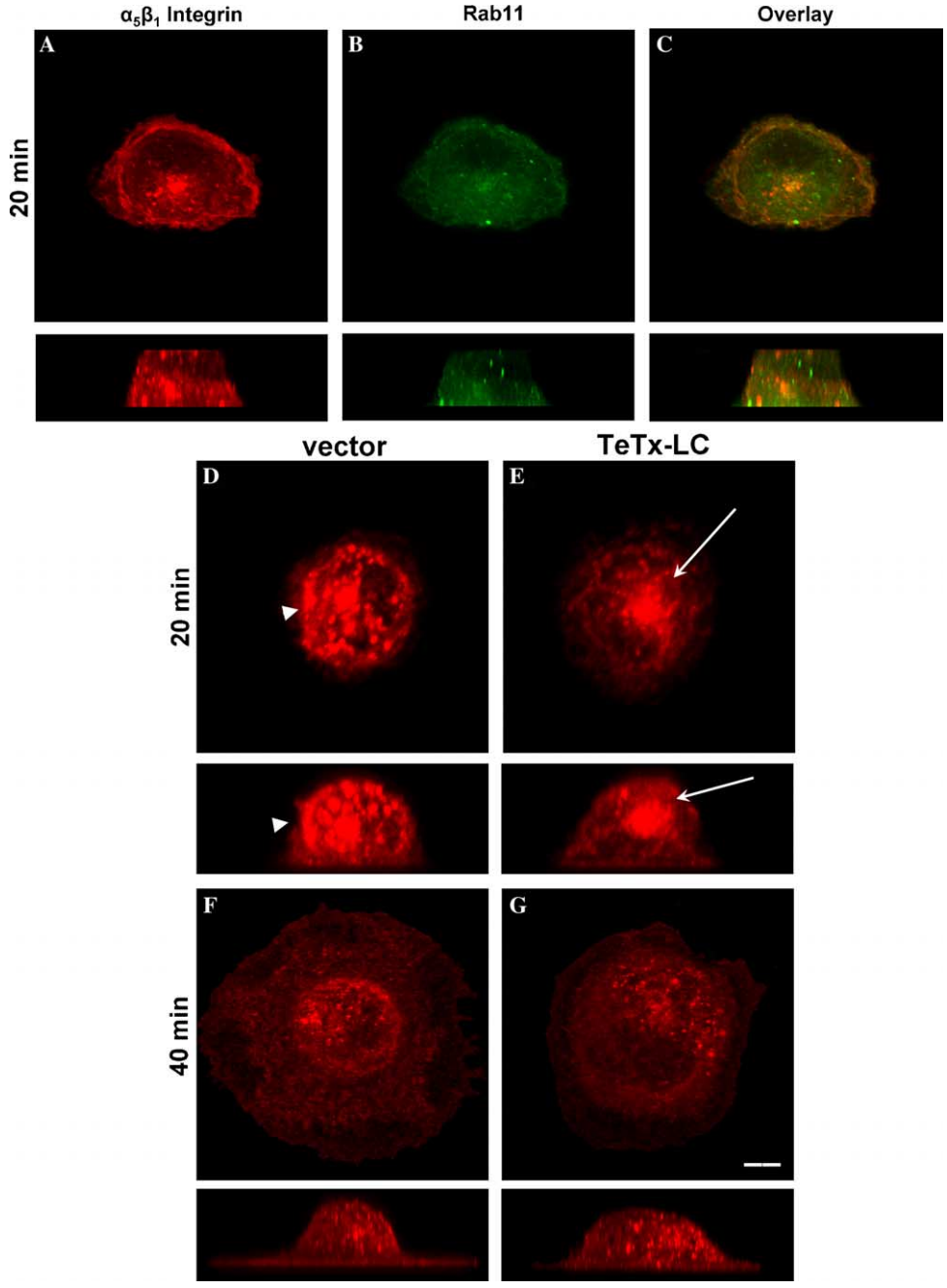

Fig. 3. TeTx-LC delays trafficking of $\alpha_5\beta_1$ integrin from a central Rab11-containing compartment. (A–C) CHO-K1 cells were serum starved for 1 h, after which $\alpha_5\beta_1$ integrin was labeled with antibody. After the cells were allowed to internalize the labeled integrins, remaining surface label was removed with trypsin. The cells were plated on FN for 20 min, fixed, permeabilized, and stained for $\alpha_5\beta_1$ integrin (A) and Rab11 (B). Overlay (C) shows co-localization of $\alpha_5\beta_1$ integrin with Rab11. (D–G) CHO-K1 cells transfected with pcDNA3.1/pEGFP (vector) or pcDNA3.1-TeTx-LC/pEGFP (TeTx-LC) for 20 h were treated as in (A)–(C) and stained for $\alpha_5\beta_1$ integrin. At 20 min, note the lack of labeled $\alpha_5\beta_1$-integrin staining in the periphery of TeTx-LC cells (E) compared to control cells (D). Arrowheads point to peripheral $\alpha_5\beta_1$ in control cells and arrows point to $\alpha_5\beta_1$ in central compartment in TeTx-LC-expressing cells. At 40 min, cells in both samples show diminished staining of the central compartment. For each image set in (A)–(G), top panels show top view; lower panels show side view after confocal 3-D reconstruction. Scale bar = 10 μm.

iated traffic in phagocytosis, secretion in mast cells, store-operated Ca^{2+} channel function, and cell migration [13,16,25,26].

We confirmed that dominant-negative NSF inhibits traffic within the endocytic-recycling pathway in the CHO cells used here and then examined the effect that E329Q-NSF had on cell attachment to FN. Inhibition of NSF blocked internalization of TMR-transferrin (Figs. 5E and F) and dramatically reduced the exocytosis of TMR-transferrin that had been internalized prior to overexpression of the dominant–negative NSF (Figs. 5G and H). In contrast to this, expression of E329Q-

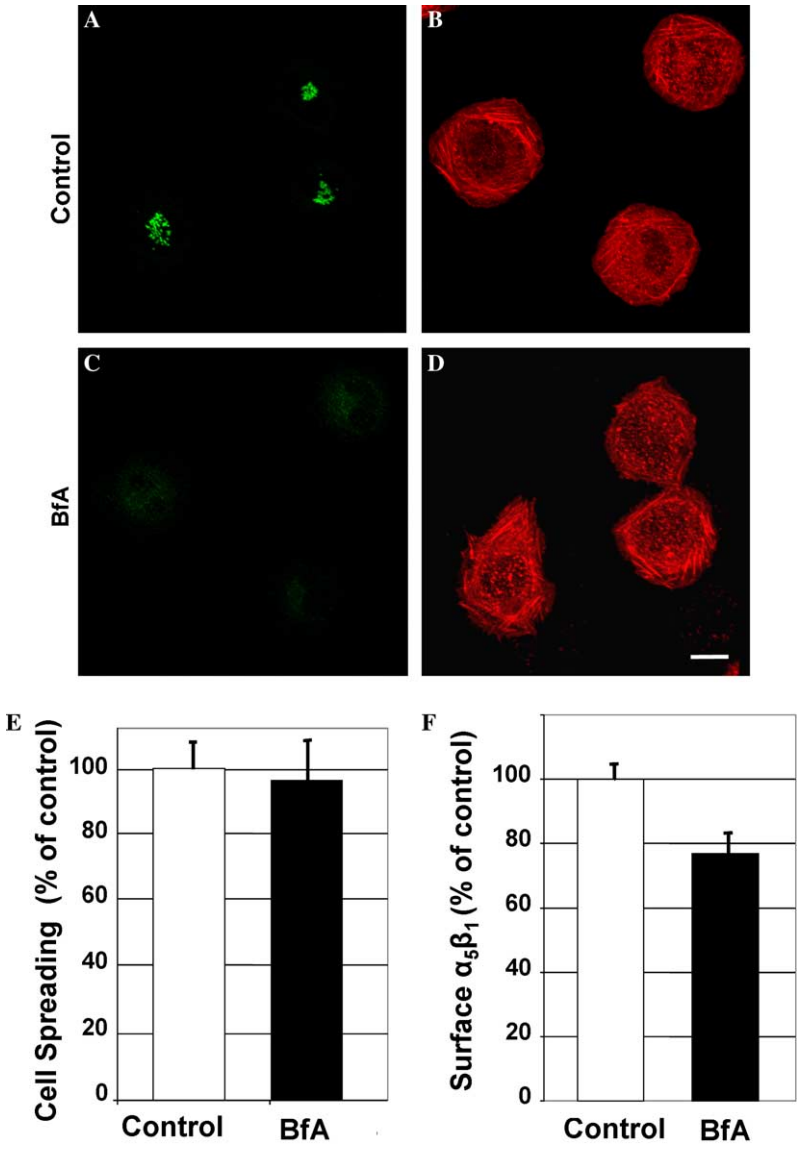

Fig. 4. BfA decreases surface expression of $\alpha_5\beta_1$ integrin, but does not alter cell spreading. (A–E) CHO-K1 cells were pretreated with 5 μg/mL BfA for 3 h. The untreated (A,B) and treated (C,D) cells were then plated onto FN-coated (20 μg/mL) coverslips in serum-free medium and incubated at 37 °C for 1.5 h. Attached cells were fixed and stained for mannosidase II (A,C) and actin (B,D). (E) The samples were microscopically examined and the ventral area of cells was quantified from digital photomicrographs using ImageJ. Results are from a minimum of three independent experiments with at least 50 cells counted per experiment, $n = 3$, means ± SEM shown. (F) CHO-K1 cells were pretreated with 5 μg/mL BfA for 3 h. The cells were then harvested at 4 °C and stained for surface $\alpha_5\beta_1$ integrin expression using a polyclonal anti-$\alpha_5\beta_1$ integrin antibody and fluorescently labeled secondary antibody. Fluorescence was quantified by flow cytometry. At least 10,000 cells were assessed per experiment, $n = 3$, mean ± SEM shown.

NSF did not alter the attachment of CHO cells to FN at 30 min (Fig. 5I) or at 90 min (data not shown), consistent with the results seen in TeTx-LC-expressing cells and confirming that SNARE-dependent membrane traffic is not required for the attachment of these cells to FN. Cell spreading was then assessed after 1.5 h of adhesion by measuring the increase in ventral cell area, post-attachment, as described above. Significantly, cells expressing E329Q-NSF had a dramatically reduced capacity (≈80% reduction) to spread compared to that of control cells (Fig. 5J). These results confirm the requirement for SNARE-mediated membrane trafficking in CHO cell spreading, distinctly from attachment, and suggest that SNAREs other than VAMP3 are involved in this process.

Inhibition of NSF alters the intracellular trafficking of $\alpha_5\beta_1$ integrin

To determine if E329Q-NSF was impairing cell spreading through a mechanism similar to that of TeTx-LC, intracellular trafficking of $\alpha_5\beta_1$ integrin was monitored as described above. CHO cells stably expressing E329Q-NSF under the control of an inducible pro-

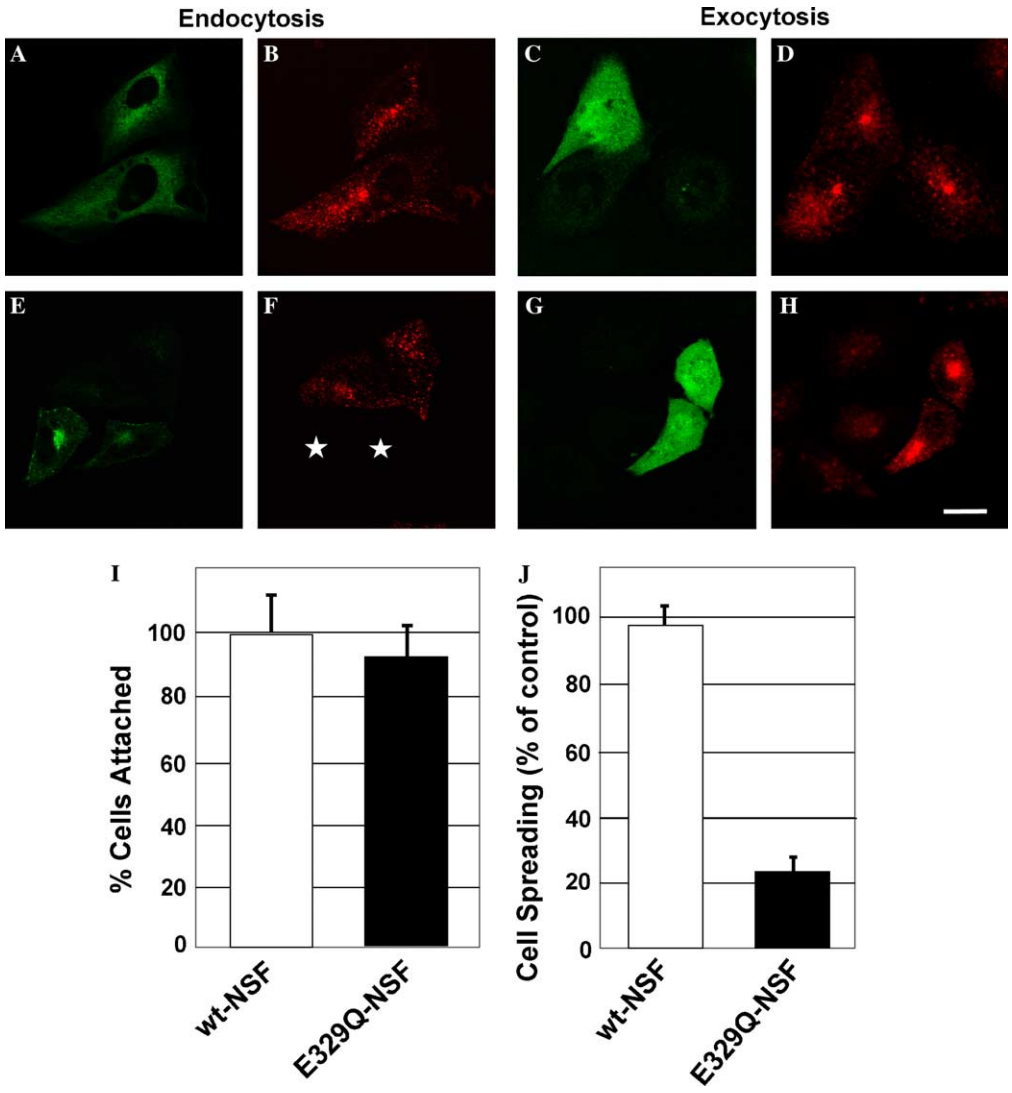

Fig. 5. E329Q-NSF inhibits cellular spreading on, but not attachment to, FN. (A,B,E,F) CHO-K1 cells were transfected with wtNSF (A,B) or E329Q-NSF (E,F) for 9 h. Cells were serum-starved for 1 h followed by incubation with 30 μg/mL TMR-Tfn for 10 min. The cells were washed to remove surface Tfn, fixed, and stained for NSF. (A,E) NSF; (B,F) TMR-Tfn. *Indicates the position of E329Q-NSF-expressing cells. (C,D,G,H) CHO-K1 cells were transfected with E329Q-NSF for 5 h and serum starved for 1 h, followed by loading with 30 μg/mL TMR-Tfn for 3 h. Cells were washed to remove surface Tfn and then incubated at 37 °C to allow exocytosis of Tfn for 0 min (E,F) or 20 min (G,H). The cells were washed, fixed, and stained for NSF. (C,G) NSF; (D,H) TMR-Tfn. Scale bar = 20 μm. (I,J) WtNSF or E329Q-NSF-expressing cells were plated onto FN-coated (20 μg/mL) coverslips in serum-free medium for 30 min (I) or 1.5 h (J). (I) Unattached cells were washed away and attached cells were fixed and stained for NSF and F-actin. The percentage of attached transfected cells was determined and quantified relative to unwashed controls. (J) Attached cells were fixed and stained for NSF expression and the increase in ventral area of transfected cells subsequent to attachment was quantified from digital photomicrographs using ImageJ. Results of at least three independent experiments are shown, with at least 50 cells counted per experiment, means ± SEM shown.

moter were examined for expression of $\alpha_5\beta_1$ by Western blot analysis. Whether induced with doxycycline to express E329Q-NSF or not, the cells showed similar levels of total cellular $\alpha_5\beta_1$ integrin (Fig. 6A). Cells transiently transfected with wtNSF or E329Q-NSF yielded comparable results (data not shown). As was the case with TeTx-LC, flow cytometry revealed that expression of E329Q-NSF decreased surface expression of $\alpha_5\beta_1$ integrin by approximately 16% compared to controls (E329Q-NSF—83.7 ± 4.3% vs. wtNSF; $n = 4$, $p \leqslant 0.05$) (Fig. 6B).

As in experiments above, the trafficking of $\alpha_5\beta_1$ integrin was examined in E329Q-NSF-expressing cells during attachment to FN by monitoring an intracellular pool of integrins labeled with fluorescent antibody. Twenty minutes after plating on FN, it was evident in untransfected (data not shown) and wtNSF transfected cells (Fig. 7A) that labeled $\alpha_5\beta_1$ integrin was clearly trafficked to the cell periphery, at sites of newly forming protrusions (arrowheads). In contrast to this, E329Q-NSF-expressing cells exhibited little or no labeled integrin at such locations; the majority of $\alpha_5\beta_1$ integrin

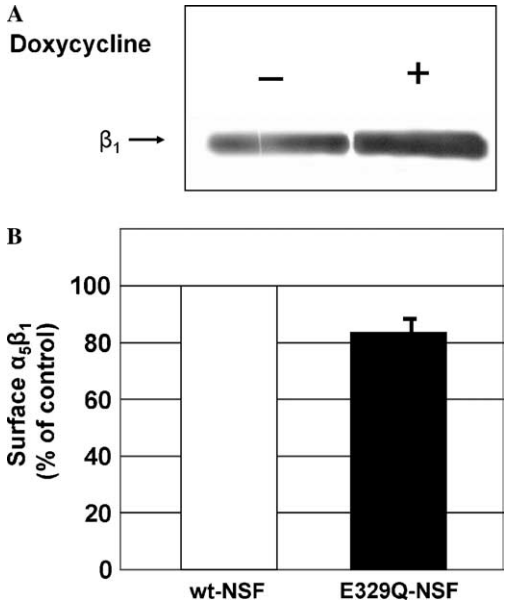

Fig. 6. E329Q-NSF decreases surface expression of $\alpha_5\beta_1$ integrin. (A) CHO-K1 cells stably transfected with E329Q-NSF under an inducible promoter were induced for 10 h and analyzed for β1-integrin expression by Western blot. (B) CHO-K1 cells were transiently co-transfected with pEGFP along with E329Q-NSF (E329Q-NSF) or wild-type NSF (wtNSF) for 10 h. The cells were then harvested and stained at 4 °C for surface $\alpha_5\beta_1$ integrin expression using a polyclonal anti-$\alpha_5\beta_1$ antibody and fluorescently labeled secondary antibody. Fluorescence of GFP-positive cells was quantified by flow cytometry. At least 5000 cells were assessed per experiment, $n = 4$, mean ± SEM shown.

remained in a central compartment, consistent with recycling endosomes (Fig. 7B, arrows). After 60 min of adhesion, it was still apparent in E329Q-NSF-expressing cells that the majority of labeled $\alpha_5\beta_1$ integrin remained trapped in a prominent central compartment (Fig. 7D). These results suggest that SNARE-mediated membrane trafficking is required for the redistribution of $\alpha_5\beta_1$ integrin from a recycling endosome to the plasma membrane, a trafficking step that is necessary for normal cellular spreading.

Discussion

In the present study, we report that inhibiting SNARE function significantly impaired spreading, but not attachment, of CHO cells on FN. VAMP3, a SNARE known to reside in the recycling endosome, was found to be required for efficient spreading; however, the effects of the general SNARE inhibitor E329Q-NSF suggest the involvement of other SNAREs as well. Importantly, we determined that SNARE activity was required for the trafficking of the FN receptor $\alpha_5\beta_1$ out of an intracellular Rab11-containing compartment to peripheral cellular locations during the process of spreading. We also report that spreading of CHO cells was not dependent on the function of BfA-sensitive Arf GEFs. Our results are consistent with studies from other laboratories, which have shown that trafficking of integrins, through intracellular compartments to the plasma membrane, is required for normal spreading and/or migration [14,27–29].

A recent study has demonstrated that inhibition of VAMP3 enhances MDCK cell attachment to ECM proteins in the presence of serum but not in its absence [14]. The study also reported that cell spreading was impaired by blocking VAMP3 function. Here, we have assessed both cell attachment and cell spreading in serum-free conditions. This was done to ensure that we were monitoring cellular events that were initiated by integrin–FN interaction. Importantly, we have also quantified the effects on cell spreading, clearly defining the role of SNARE function subsequent to cell attachment to ECM substrate. Furthermore, the fact that dominant-negative NSF was a more potent inhibitor of spreading than TeTx-LC (Figs. 1 and 5) suggests that SNARE-dependent membrane trafficking pathways, other than those regulated by VAMP3, are involved in cell spreading. These pathways would have been inhibited by disruption of NSF function but not by specifically blocking VAMP3 activity. We are currently working to identify additional SNARE proteins that are involved in cell adhesion.

The use of dominant-negative NSF as a general inhibitor of SNARE-mediated membrane traffic has been well documented [16,30,31]. To assess the contributions to cell adhesion made by different paths of intracellular membrane traffic, we examined the role of the biosynthetic-secretory pathway during CHO cell spreading. The observation that BfA did not alter cell spreading is in apparent disagreement with evidence, suggesting that BfA inhibits lamellipodium extension [32]. It should be noted, however, that we have used BfA treatment periods that are much shorter (3 h vs. 18 h) than those used in the previous study [32]. Consistent with work published by other laboratories [23,33,34], we confirmed that under our conditions BfA caused disruption of the Golgi apparatus, and presumably membrane trafficking within the biosynthetic-secretory pathway, by staining cells for the Golgi marker mannosidase II. Therefore, we conclude that spreading in CHO cells is regulated by SNARE-mediated trafficking pathways, including a VAMP3-dependent trafficking pathway, but that these pathways are not BfA-sensitive.

One possible explanation for the diminished spreading of cells observed here is the decrease in the level of cell surface integrin that was induced by TeTx-LC or E329Q-NSF. This is likely to be only a partial explanation since TeTx, E329Q-NSF, and BfA all reduced surface $\alpha_5\beta_1$ integrin levels to the same extent (≈15–20%), yet these three inhibitors produced significantly different effects on cell spreading. This suggests that the

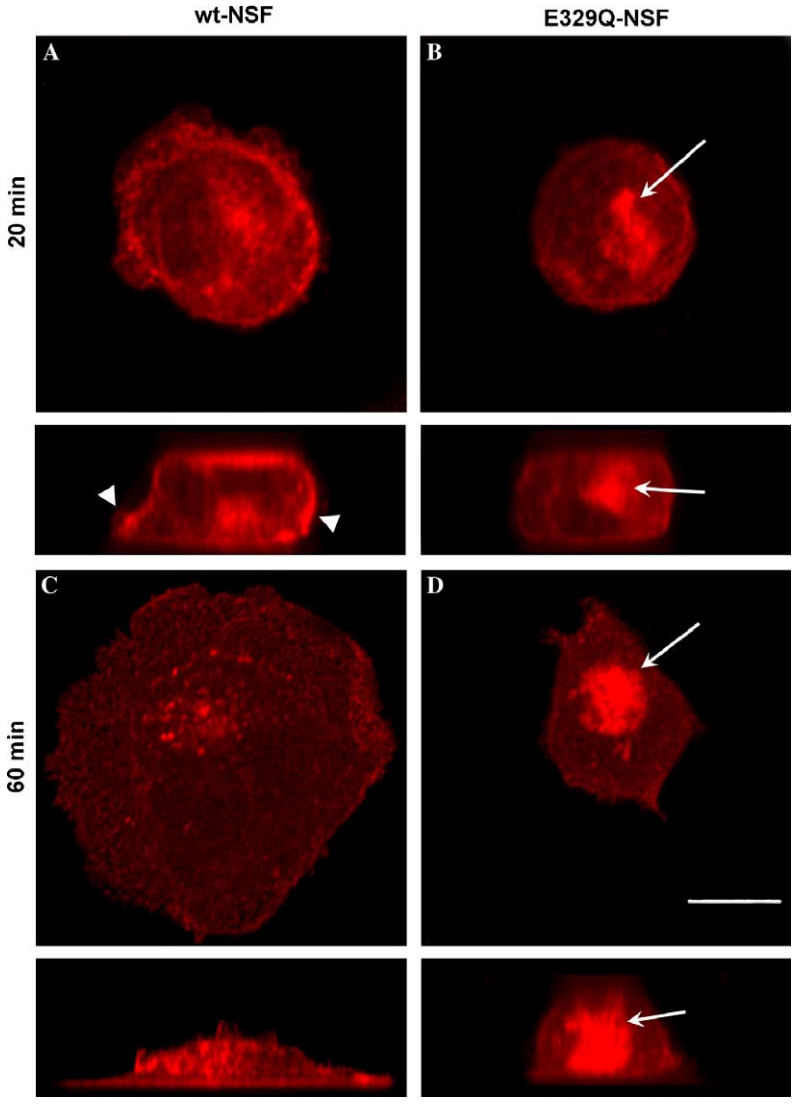

Fig. 7. E329Q-NSF inhibits trafficking of $\alpha_5\beta_1$ integrin out of a central intracellular compartment. CHO-K1 cells, transfected with E329Q-NSF for 5 h, were serum starved for 1 h. Anti-integrin antibody (1:100) diluted in DMEM was applied for 3 h. After incubation at 37 °C to allow internalization of the labeled integrins, the remaining surface label was removed with trypsin. The cells were plated on FN for 20 min (A,B) or 60 min (C,D), then fixed, permeabilized, and stained for NSF expression and $\alpha_5\beta_1$. (A,C) $\alpha_5\beta_1$ staining in wt-NSF-expressing cells. (B,D) $\alpha_5\beta_1$ staining in E329Q-NSF-expressing cells. Arrowheads in (A) point to $\alpha_5\beta_1$ that is located in the periphery of the cell. Arrows in (B) and (D) point to $\alpha_5\beta_1$ located in prominent, central compartments. Scale bar = 10 μm.

defect in cell spreading was not simply due to changes in absolute levels of cell surface integrin. It is therefore reasonable to propose that the intracellular trafficking of a particular population of integrins has been differentially altered by the different inhibitors. Recent evidence points to the existence of functionally important populations of integrin receptors within cells. For example, Pierini et al. [35] were able to show that integrins are trafficked to the leading edges of lamellipodia, where they were proposed to be critical for anchoring of the membrane to the ECM. Furthermore, a population of high-affinity integrins has been shown to be delivered to sites of lamellipodium protrusion [36]. Thus, the putative integrin recycling pathway that is impaired by TeTx-LC and E329Q-NSF in the present study might be required for the redistribution of a population of integrins that is needed to form new adhesions at sites of membrane extension and thereby contribute to the stability of nascent lamellipodia. In contrast, treatment of cells with BfA might perturb the delivery of a different population of integrins (perhaps, newly synthesized) to the cell surface. This population of integrins may not contribute directly, or immediately, to lamellipodium formation and therefore may not significantly affect cell spreading.

To examine changes in the intracellular compartmentalization of integrins, we monitored the distribution of integrins within cells during adhesion. In

control and wtNSF-expressing cells, integrins were trafficked from a central compartment to sites of membrane protrusion. We have demonstrated that the integrin compartment contains Rab11, a marker that is indicative of recycling endosomes. In TeTx-LC expressing cells, trafficking of $\alpha_5\beta_1$ integrin out of this compartment during spreading was delayed. More dramatic than the effect of TeTx was the effect that E329Q-NSF had upon integrin trafficking out of the Rab11 compartment. This is a possible explanation for the observation that E329Q-NSF is a more potent inhibitor of spreading than TeTx-LC. E329Q-NSF appears to trap $\alpha_5\beta_1$ integrin within a recycling compartment, while TeTx-LC delays redistribution of this integrin, possibly until a less efficient or indirect SNARE-dependent trafficking pathway compensates. These results are consistent with the notion that inhibiting NSF function will impair all SNARE-mediated traffic in the cell, including that blocked by TeTx-LC. The effect of E329Q-NSF is similar to that seen by Powelka et al. [12] where a dominant negative mutant of Rab11 impaired the exocytosis of β_1 integrin from a Rab11-containing compartment and inhibited cell migration.

An alternative explanation for our findings is that, as well as the redistribution of integrins, SNARE-mediated membrane traffic is required for the delivery of other proteins or membrane components that regulate cell adhesion. For example, the targeted delivery of focal adhesion proteins may be disrupted by inhibition of SNARE function, leading to altered adhesive or signaling properties within focal adhesions. Intracellular trafficking of signaling components could involve several different trafficking pathways and therefore might also contribute to the differential effects that TeTx and dominant-negative NSF have upon cell adhesion. Such alterations could impact integrin-regulated remodeling of actin structures, reducing lamellipodium extension and/or stress fiber formation. In consideration of these possibilities, we have determined that expression of dominant-negative NSF does not alter the number of focal adhesions formed in CHO cells [13] and we have not detected any changes in the actin cytoskeleton of cells as a result of blocking SNARE-mediated traffic (data not shown).

In summary, the results of the present study are consistent with a model of cell adhesion in which the membrane trafficking of integrins, and possibly other proteins involved in adhesion, occurs through specific intracellular compartments in support of efficient cell spreading. Our studies quantitatively define the requirement of VAMP3 in spreading but, importantly, indicate that membrane trafficking pathways mediated by other SNAREs are also involved in this process. Future studies will identify these SNAREs and characterize their roles in integrin trafficking and signaling during the regulation of cell adhesion.

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