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Inhibition of SNARE-mediated membrane traffic impairs cell migration

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Abstract

Cell migration occurs as a highly-regulated cycle of cell polarization, membrane extension at the leading edge, adhesion, contraction of the cell body, and release from the extracellular matrix at the trailing edge. In this study, we investigated the involvement of SNARE-mediated membrane trafficking in cell migration. Using a dominant-negative form of the enzyme N-ethylmaleimide-sensitive factor as a general inhibitor of SNARE-mediated membrane traffic and tetanus toxin as a specific inhibitor of VAMP3/cellubrevin, we conducted transwell migration assays and determined that serum-induced migration of CHO-K1 cells is dependant upon SNARE function. Both VAMP3-mediated and VAMP3-independent traffic were involved in regulating this cell migration. Inhibition of SNARE-mediated membrane traffic led to a decrease in the protrusion of lamellipodia at the leading edge of migrating cells. Additionally, the reduction in cell migration resulting from the inhibition of SNARE function was accompanied by perturbation of a Rab11-containing $\alpha_5\beta_1$ integrin compartment and a decrease in cell surface $\alpha_5\beta_1$ without alteration to total cellular integrin levels. Together, these observations suggest that inhibition of SNARE-mediated traffic interferes with the intracellular distribution of integrins and with the membrane remodeling that contributes to lamellipodial extension during cell migration.

Keywords: Cell migration; Integrin; Membrane traffic; NSF; SNARE; VAMP3

Introduction

Directed cell migration is critical to many physiological and pathological processes including embryonic development, wound healing, angiogenesis, and tumor metastasis [1–3]. Migration occurs through a series of dynamic interactions between the cell and the extracellular matrix (ECM) and can be broken down into a series of steps: cell polarization, membrane extension at the leading edge, adhesion, contraction of the cell body, and release from the ECM at the trailing edge [4,5]. In sequence, these steps result in the net translocation of the cell in a defined direction.

While a step-wise model of cell migration is conceptually simple, at the cellular level migration is a complicated, highly regulated process and many types of proteins have been assigned important functions in its control. Integrins are transmembrane receptors for components of the ECM that provide a link between the ECM and the cytoskeleton. This linkage is generally effected through a series of scaffolding proteins, for example, paxillin and talin, and helps to localize the exertion of actinomyosin-generated forces against the substrate [6,7]. The small GTPases Rac and Cdc42, which regulate the actin cytoskeleton to induce the formation of lamellipodia and fillipodia, respectively, are instrumental in controlling the polarization (Cdc42) and the protrusion of the advancing edge (Rac) that are observed in migrating cells [8–10]. Cytoskeletal regulatory proteins such as the actin-nucleating Arp2/3 complex and WASP family proteins control actin polymerization at the leading

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edge, thereby modulating the protrusive force necessary for membrane extension [11,12]. Ultimately, the activities of all of these proteins are finely orchestrated, both temporally and spatially, to control the rate and direction of migration.

Migrating cells typically have well-defined leading edges that are characterized by extensive actin remodeling as well as the formation of nascent focal adhesions. In addition to this, migrating cells exhibit a constant flow of membrane proteins from the leading edge back towards the centre of the cell [13,14]. The highly polarized morphology and retrograde flow that typify migrating cells suggest the requirement for targeted delivery of membrane components to the front of the cell. Previous studies have shown that integrins on migrating cells are rapidly endocytosed at the trailing edge and are subsequently exocytosed at the leading edge [15–17]. Much recent evidence now indicates that intracellular trafficking of integrins in motile cells is a highly regulated process involving the activities of PKC [18], PKD [19,20], PKB/Akt [21], ARF6 [22], and Rab proteins [16,22]. Collectively, these data strongly suggest that regulated membrane traffic is required to provide a supply of membrane components, including integrins and possibly additional scaffolding and signaling proteins, to the leading edge for the formation of new adhesive contacts.

Intracellular membrane traffic consists of a series of coordinated steps that allow the vesicular transport of cargo proteins to specific locations in the cell. Most trafficking is mediated by the activity of soluble NSF-attachment protein receptors (SNAREs) found on the vesicle and target membranes. The restricted subcellular distribution of most SNARE proteins and the restricted pairing of SNAREs between vesicle and target membranes, as well as other regulatory mechanisms including the presence of tethering factors, combine to ensure that the delivery of cargo is directed to specific subcellular compartments or regions of the plasma membrane [23,24]. After the fusion of vesicles with target membranes, SNARE complexes are disassembled for recycling of the SNARE proteins. This is accomplished via the activity of the ATPase/chaperone N-ethylmaleimide-sensitive factor (NSF), which is a key regulator of much of the intracellular membrane traffic within eukaryotic cells [25,26]. While much is known about intracellular membrane traffic in cellular processes such as protein secretion and phagocytosis, the molecules that directly mediate membrane traffic to facilitate cell migration have not been characterized.

Given the putative role of intracellular membrane traffic in cell motility, we examined the function of SNARE-mediated membrane traffic in directed cell migration. A dominant-negative mutant form of NSF (E329Q-NSF) was used as a general inhibitor of membrane fusion to demonstrate the requirement for SNARE-mediated membrane traffic in cell migration. Expression of the catalytic light chain of Tetanus toxin was used to specifically test the involvement of VAMP3/cellubrevin in cell migration. We report that cell migration is dependent upon both a VAMP3-mediated trafficking pathway and a VAMP-3-independent pathway. Importantly, we present evidence that the defect in migration caused by inhibition of membrane traffic results from both a reduction in the level of integrin on the cell surface and a disruption of membrane remodeling that leads to impaired lamellipodial extension.

Materials and methods

Reagents, cell culture, and transfection

CHO-K1 cells were cultured in DME with 10% fetal bovine serum. CHO-K1 cells stably transfected with either wildtype or E329Q NSF were constructed using the Tet-ON inducible expression system (BD Biosciences, Mississauga, ON) and cultured in DME with 10% fetal bovine serum, hygromycin (BD Biosciences) and G418 (BD Biosciences). Rabbit polyclonal antibody against VAMP3 was obtained from Affinity Bioreagents (Golden, CO). Mouse monoclonal antibody against paxillin was purchased from Upstate Biotechnology (Lake Placid, NY). Goat polyclonal antibody against TeTx was obtained from Biodesign International (Saco, ME) and rabbit anti-β-tubulin polyclonal antibody was from Sigma (Oakville, ON). Rabbit anti-Rab11 antibody was from Stressgen (San Diego, CA). Rabbit polyclonal antibody against Mannosidase II was a kind gift of Dr. M Farquhar (University of California at San Diego, CA). Rhodamine-labeled phalloidin, and AlexaFluor 488 and AlexaFluor 594-conjugated secondary antibodies were purchased from Molecular Probes (Eugene, OR).

pEGFP-N1 was purchased from CLONTECH Laboratories Inc. (Palo Alto, CA). Wild-type and E329Q NSF constructs in pcDNA3.1 were described previously [27]. Tetanus toxin light chain in pcDNA3.1 and TR-VAMP3-GFP were a kind gift of Dr. William Trimble (Hospital for Sick Children, Toronto). CHO-K1 cells were transiently transfected with the indicated plasmid DNA using FuGene6 transfection reagent according to the manufacturer's protocol (Roche Applied Science, Laval, QC).

Trans-well migration assays

The lower side of polycarbonate membranes with 8 mm pores (Neuroprobe, Gaithersburg, MD) were coated with 20 μg/mL bovine fibronectin (Sigma, Oakville, ON) for 3 h at 37°C. Cells were harvested using 5 mM EDTA/PBS, resuspended in serum-free DME and counted using a hemocytometer. The lower wells of an AP48 transwell migration chamber (Neuroprobe, Gaithersburg, MD) were filled with DME with 10% fetal bovine serum and covered with the coated membrane. Cells in serum-free DME were added to the top chamber at 2000 cells/mm^2 of membrane and the chamber was placed at 37°C, 5% CO_2, 100% RH for 4.5 h. The membrane was removed and cells fixed in 4% paraformaldehyde for 20 min. Cells were removed from

either the top or bottom of the membrane using a rubber wiper.

Transwell cell migration was quantitated in one of two ways. In assays using stably transfected NSF-expressing cells, cells on the membrane were stained using 0.4% (w/v) sulfarhodamine B (Sigma, Oakville, ON) in 1% acetic acid for 30 min, followed by 3 washes with 1% acetic acid. After air drying, the dye was eluted using 10 mM unbuffered Tris and quantified at 570 nm using a visible-light plate reader. In all other assays, cells on the membrane were stained with 300 nM DAPI in PBS (Sigma, Oakville, ON) for 5 min, washed twice in PBS and the membranes mounted on microscope slides using DAKO Fluorescent mounting media (DAKO Cytomation, Carpinteria, CA). Cells were enumerated using epifluorescence microscopy. For statistical analyses of migration assay data, the Student's *t* test was applied.

Immunoblotting

Cells were harvested in 5 mM EDTA/PBS and lysed using lysis buffer (1% Triton X-100, 0.1% SDS, 0.5% deoxycholic acid in PBS) for 20 min at 4°C. Lysate was spun at 13000 RPM for 10 min and 30 μg of supernatant protein was electrophoresed through a 12% polyacrylamide gel and transferred to PVDF membrane (Pall Life Sciences, Mississauga, ON). The membranes were blocked in 5% skim milk powder in TBS with 0.1% Tween-20 (TBS-T) and probed with primary antibody (rabbit anti-VAMP1, VAMP2 or VAMP3) diluted in TBS-T followed by HRP-conjugated secondary antibody at 1:5000 dilution. Bound antibodies were detected by ECL Plus chemiluminescence (Amersham Biosciences, Piscataway, NJ).

Immunofluorescence microscopy

Cells growing on fibronectin coated coverslips were fixed using 4% paraformaldehyde in PBS for 20 min, quenched in 0.1 M glycine in PBS for 20 min and permeabilized with 0.2% Triton X-100 in PBS. After washing with PBS, cells were blocked in 5% skim milk powder in PBS. Primary antibody was diluted in PBS and added to the cells for 1 h. After washing the cells five times with PBS, secondary antibody and/or rhodamine-phalloidin diluted 1:5000 in 5% skim milk in PBS was added for 1 h. Coverslips were mounted on slides using DAKO Fluorescent mounting medium and viewed using a confocal imaging station equipped with a Leica DM-IRE2 inverted microscope. Images were taken using a Leica 63× oil immersion lens and pseudocolored using LCS Software.

Quantitation of surface integrin

Cells to be stained for surface integrin were transfected and/or treated as outlined above. Cells were harvested using 5 mM EDTA/PBS and resuspended in ice-cold EDTA/PBS supplemented with 0.5% BSA (wash buffer). After harvesting, cells were kept cold to prevent endo- or exocytosis, which would alter surface levels of integrins. Cells were counted using a hemocytometer and diluted in wash buffer such that all samples were at the same concentration. Cells were then pelleted at 400 × *g* for 2 min at 4°C, resuspended in 500 mL of goat anti-α5β1 antibody (Chemicon, Temecula, CA) diluted 1:100 in wash buffer and incubated on a rotator for 1–1.5 h at 4°C. Cells were pelleted and washed with wash buffer five times and stained with AlexaFluor 594-conjugated rabbit anti-goat secondary antibody (Molecular Probes, Eugene, OR) in the same manner as the primary antibody. After removal of the secondary antibody, the cells were washed three times in wash buffer and run on a Coulter flow cytometer to quantitate fluorescence. Forward and side-scatter gates were positioned so as to include only single, whole cells. A minimum of 2000 positive cells were counted for each experiment.

Polarization and protrusion assays

Confluent cell monolayers growing on fibronectin-coated coverslips were wounded using a 200-μL pipette tip. After washing briefly with medium to remove loosened cells, the coverslips were either fixed immediately using 4% paraformaldehyde or incubated at 37°C, 5% CO_2, 100% RH for 2 h and then fixed. Cells were stained and mounted for immunofluorescence microscopy as described earlier. Using a confocal imaging station equipped with a Leica DM-IRE2 inverted microscope, cells along the wound edge were scored for polarization based on the position of the MTOC relative to the nucleus. In a method similar to that described by Prigozhina and Waterman-Storer [20], the MTOC was recorded as being either between the wound edge and the nucleus, to the side of the nucleus or behind the nucleus, away from the wound edge. At least 50 cells were scored per experiment. Cells with the MTOC towards the wound were considered polarized. To observe cell protrusion into a wound, the actin cytoskeleton was stained using rhodamine-phalloidin as described earlier. For quantification of focal adhesions, confocal images of paxillin-stained cells were acquired and imported into ImageJ software (NIH) for analysis. Paxillin-containing structures between 0.5 and 50 μm^2 were counted.

Endocytic trafficking assay

Cells were grown on glass coverslips and serum-starved overnight in DMEM. Anti-$\alpha_5\beta_1$ antibody was applied to the cells, which were incubated at 37°C for 3 h to allow internalization of the antibody. Cell surface antibody was then removed with trypsin. To stimulate trafficking of integrins, the cells were incubated in 10% FBS at 37°C for 10 min. Samples were fixed, permeabilized, stained with antibodies to Rab4 or Rab11 followed by fluorescently

labeled secondary antibodies and then imaged as described above.

Results

Expression of E329Q-NSF reduces cell migration

Previous studies have indicated that adhesion molecules are delivered to the leading edge of migrating cells in a polarized fashion [17,28]. The delivery of this material can occur through biosynthetic-secretory pathways and through endocytic recycling pathways [15,22,29,30]. Since both of these types of pathways are putatively dependent upon SNARE-mediated membrane traffic, we directly examined the involvement of SNARE function in the migration of CHO cells by expressing E329Q NSF, a dominant negative form of the ATPase NSF. NSF is the enzyme that is primarily responsible for disassembly of SNARE complexes in mammalian cells. As such, inhibition of this enzyme eventually results in the accumulation of SNARE complexes within cells and the elimination of free SNAREs that can support vesicle mediated membrane traffic [31]. The efficiency with which, and the mechanism by which, E329Q NSF impairs SNARE-mediated membrane traffic have been described in detail previously [27,32].

The ability of cells expressing either wild-type or E329Q NSF (under the control of an inducible promoter) to migrate was assayed using a transwell migration assay. After 4.5 h of migration, it was found that cells expressing E329Q NSF exhibited a 35.3% reduction in migration when compared to wild-type NSF-expressing control cells (Fig. 1), indicating that SNARE-mediated membrane traffic is required for normal cell migration. Expression of exogenous wildtype NSF did not significantly change cell migration from that of parental cells (data not shown).

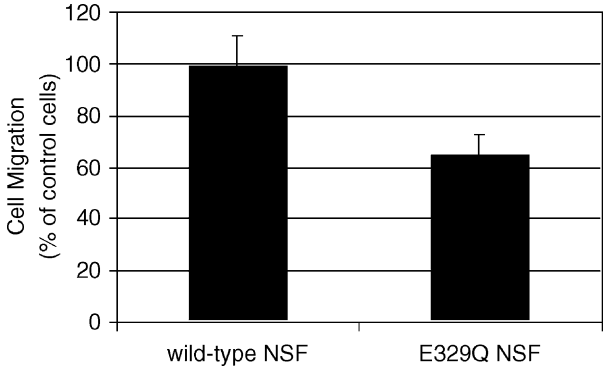

Fig. 1. Expression of dominant-negative NSF impairs haptotactic migration in CHO-K1 cells. Expression of wild-type or mutant NSF (E329Q-NSF) in stably transfected cell lines was induced by addition of doxacycline for 6 h. Cell migration assays were performed using a transwell chamber containing polycarbonate membranes coated on the underside with fibronectin. Cells were allowed to migrate for 4.5 h, the membranes were fixed and then cells on the lower side of the membrane were stained with 0.4% w/v sulforhodamine B. After air-drying, the absorbed dye was eluted and quantified spectrophotometrically at 590 nm. Data are reported as the mean percentage of the uninduced control ($n = 6$, $P < 0.05$).

E329Q-NSF expression impairs protrusion of lamellipodia

To determine the point at which blocking NSF function interferes with cell migration, we examined specific steps in the migration process. It is generally accepted that directed migration begins with cell polarization and it has been shown in previous studies that the microtubule organizing centre (MTOC) and the Golgi apparatus are reoriented towards the leading edge of cells in wound-healing migration assays [33,34]. We assessed the effect of inhibition of SNARE-mediated membrane traffic on cell polarization by examining the reorientation of the MTOC in cells at the edge of a scratch wound. Cells expressing wildtype or E329Q NSF were scored based on the position of the MTOC relative to the nucleus. Cells with the MTOC on the wound side of the nucleus were considered polarized. As a control, untreated cells were fixed immediately after wounding; in these cells, the MTOC appeared randomly oriented in the cells. In cells incubated for 2 h after wounding, the MTOC was oriented towards the wound in approximately 70% of untreated cells as well as those expressing wildtype NSF or E329Q NSF. These observations indicate that SNARE-mediated membrane traffic is not required for cell polarization (Fig. 2).

Given that cell polarization is normal after inhibition of SNARE-mediated membrane trafficking, we examined the effect of such inhibition on the ability of cells to form directional protrusions. After wounding cell monolayers and incubating as described above, samples were fixed, stained using rhodamine-phalloidin, and then microscopically examined for cells that had formed actin-containing lamellipodia which protruded into the wound. As depicted in Fig. 3, cells expressing E329Q-NSF were unable to form prominent lamellipodia that attached to the substratum in the wound. This is in direct contrast with control cells, which typically contained large protrusions extending into the wound (Fig. 3).

Expression of E329Q-NSF decreases cell surface integrin levels but not the number of focal adhesions

Previous studies suggest that integrins are recycled to the leading edge of the cell during cell migration to facilitate contact with the extracellular matrix, stabilizing lamellipodia and supporting the generation of motile forces [15,17,22]. We therefore speculated that the reduction in cell migration caused by the inhibition of membrane trafficking might be due to a reduction in the number of integrins reaching the cell surface. To evaluate the requirement for SNARE-mediated traffic in cell surface integrin expression, we quantified the level of cell surface fibronectin receptor ($\alpha5\beta1$ integrin) in control and E329Q-NSF-expressing CHO cells by flow cytometry.

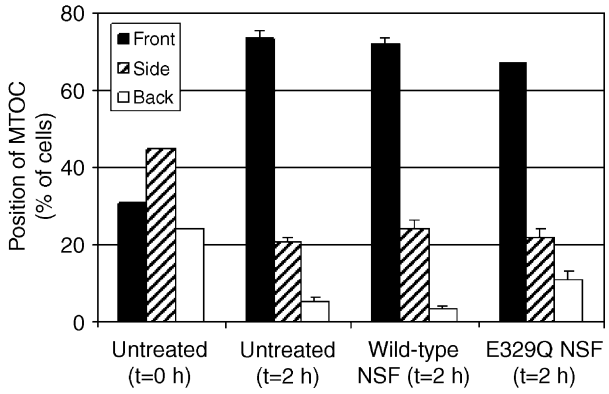

Fig. 2. Inhibition of membrane traffic by E329Q-NSF does not inhibit cell polarization. Cells were transiently transfected with wild-type NSF or E329Q-NSF. After 6 h, cells were harvested and plated at near confluency on fibronectin-coated coverslips. Two hours after plating, monolayers were wounded. Cells were fixed immediately (0 h) or 2 h after wounding and were stained with antibodies to NSF and β-tubulin. Nucleii were stained using DAPI (300 nM). The position of the MTOC relative to the nucleus in cells along the wound edge was scored as being either towards the wound (Front), away from the wound (Back) or neither (Side). Data are the results of at least 3 independent experiments.

Cells expressing mutant NSF exhibited a 16.3% drop in surface integrin when compared to those expressing wild-type NSF (Fig. 4). Expression of wildtype NSF did not significantly alter surface integrin levels from those observed in parental CHO cells (data not shown). Western blot analyses based on equal amounts of protein from cell extracts indicated that E329Q-NSF expression did not alter

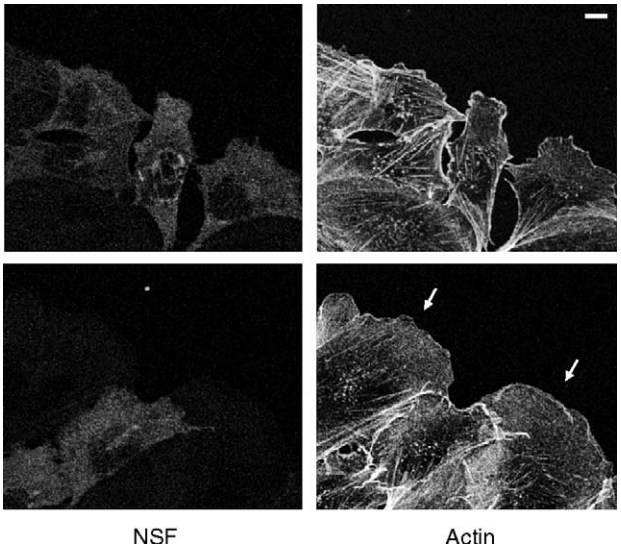

Fig. 3. Inhibition of membrane traffic by E329Q-NSF inhibits the formation of lamellipodia. CHO cells were transiently transfected with E329Q-NSF. After three hours, cells were lifted and re-plated at near confluency on fibronectin-coated coverslips. Cell monolayers were wounded 3 h after plating, incubated at 37°C for 4 h and then fixed. Cells were stained with anti-NSF antibody and F-actin was labeled with rhodamine-phalloidin. Top panels show three E329Q-NSF-transfected cells at the wound edge. For comparison, the bottom panels show an E329Q-NSF transfected cell between 2 untransfected cells. Arrows indicate normal lamellipodia in untransfected cells. Scale bar = 10 μm.

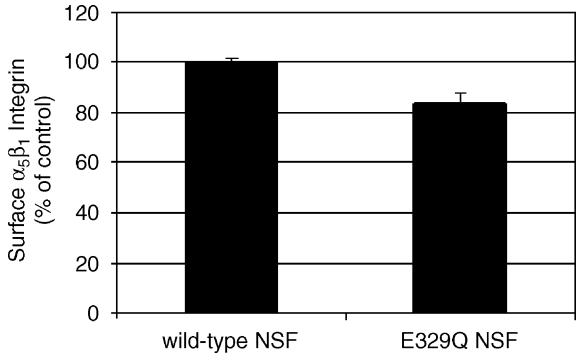

Fig. 4. Expression of E329Q-NSF reduces the level of cell surface integrin. CHO-K1 cells were co-transfected with wildtype NSF or E329Q-NSF together with GFP at a 10:1 molar ratio. After 10 h, cells were harvested and surface integrin was fluorescently labeled using goat anti-fibronectin receptor antibody followed by Alexa 595-conjugated rabbit anti-goat secondary antibody. Alexa-594 fluorescence of cells expressing GFP was quantified on a Coutler flow cytometer. Data are the result of at three independent experiments ($P < 0.01$).

total cellular levels of α5β1 integrin (data not shown). Taken together, these data suggest that SNARE-mediated membrane trafficking may contribute to cell migration by delivering integrins to the cell surface.

The ability of E329Q-NSF to lower levels of cell surface integrin and impair lamellipodial protrusion suggested that a decrease in the efficiency of cell-ECM contact might be impeding the formation of lamellipodia. We reasoned that a decrease in the number of integrin receptors might impair the ability of membrane protrusions to attach to the substratum and this might also be reflected in a change in the number of focal adhesions in E329Q-NSF-expressing cells. To investigate this possibility, we used wound-healing assays to examine cells for paxillin-containing focal adhesions. We found that E329Q-NSF-expressing cells contained a similar number of paxillin-containing focal adhesions to untransfected or vector-transfected control cells (Fig. 5). Control cells tended to have larger lamellipodia containing proportionately more focal adhesions (see Fig. 5); however, quantitative image analysis determined that the number of focal adhesions per unit cell area was not significantly different (Fig. 5E).

Cleavage of VAMP3 with tetanus toxin reduces cell migration

The reduction in migration observed after expression of E329Q NSF indicates that SNARE-mediated membrane traffic is involved in cell migration. SNAREs are involved in a large number of trafficking pathways throughout the cell and, while all of these pathways are likely to be blocked by inhibition of NSF function, it is probable that only a subset of these pathways is directly involved in cell migration. In order to identify specific trafficking pathways that are involved in cell migration, more specific inhibitors were used. Previous studies have shown that VAMP3 is a resident of the recycling endosome and can localize to the

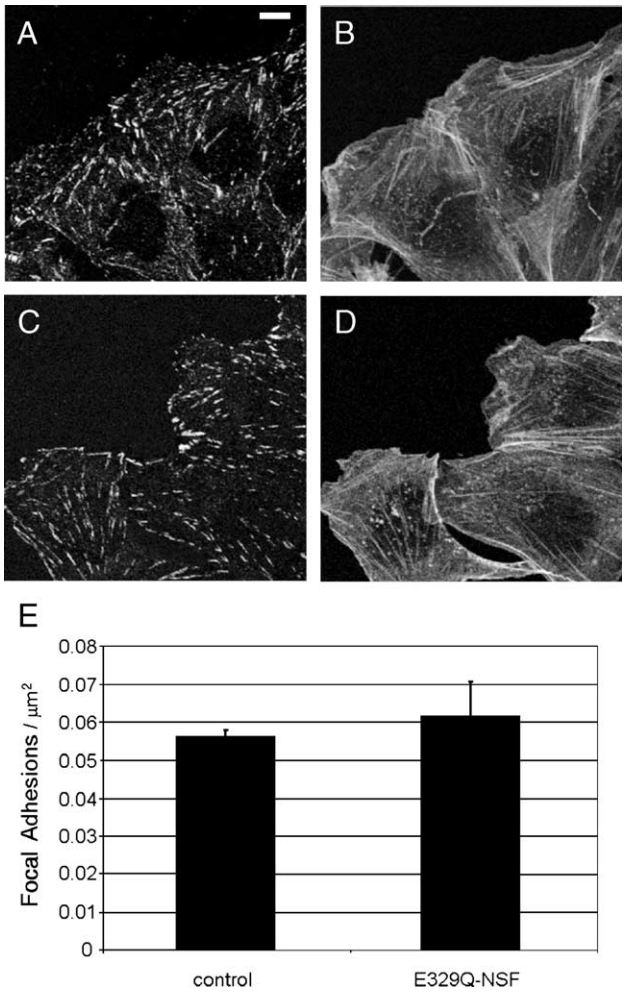

Fig. 5. Inhibition of membrane traffic by E329Q NSF does not alter the number of focal adhesions in CHO-K1 cells. Expression of NSF was induced in wild-type (A and B) and E329Q-NSF-expressing (C and D) cell lines by the addition of doxacycline. Three hours after induction, cells were plated on fibronectin-coated coverslips at near confluency. Monolayers were wounded 3 h later and then incubated at 37°C for 4 h. Cells were fixed and stained with anti-Paxillin antibody (A and C) and F-actin was labeled with rhodamine-phalloidin (B and D). Scale bar = 10 μm. (E) Focal adhesions in the area between the nucleus and wound edge of individual cells were counted using ImageJ software and the number of focal adhesions per μm^2 was determined. Data are the results of 3 independent experiments.

plasma membrane where it participates in exocytic vesicle fusion, for example, during transferrin-receptor recycling and phagosome formation [35–37]. We thus hypothesized that VAMP3 may also function to regulate recycling membrane traffic in support of cell migration.

To examine the involvement of VAMP3-mediated vesicular traffic in cell migration, the catalytic light chain of tetanus toxin (TeTx), a protease that cleaves and inactivates VAMP1, VAMP2, and VAMP3, was expressed in CHO cells. Western blot analysis determined that neither VAMP1 nor VAMP2 is expressed at detectable levels in CHO-K1 cells (Fig. 6A). In lysates from cells expressing TeTx, VAMP3 was undetectable, suggesting that the cleavage of this SNARE by the toxin was complete. In transwell migration assays, cells expressing TeTx showed a 19.5% reduction in migration compared to cells transfected with empty vector (Fig. 6C).

To confirm a role for the VAMP3 protein in cell migration, a vector containing the cDNA for the light chain of TeTx was co-transfected into CHO cells along with a TeTx-resistant VAMP3-GFP fusion (TR-VAMP3-GFP) protein. Western blot analysis for VAMP3 expression in lysates from transfected cells revealed that cleavage of VAMP3 was complete in GFP transfected cells. Importantly, in cells transfected with TR-VAMP3-GFP, only the endogenous VAMP3 was cleaved; the TeTx-insensitive VAMP3-GFP fusion was unaffected (Fig. 6B). Using migration assays as described above, we determined that co-expression of TR-VAMP3-GFP with TeTx was able to fully rescue migration (Fig. 6C). Co-expression of TR-VAMP3-GFP with an empty vector did not change cellular migration compared to cells expressing GFP alone. These results suggest that CHO cell migration is partially dependent on a VAMP3-mediated membrane trafficking pathway.

The observation that TeTx reduced migration less dramatically than E329Q-NSF (19.5% reduction vs. 35.3%, respectively) prompted us to examine how TeTx would affect integrin expression. Surprisingly, the expression of TeTx decreased cell surface integrin expression to a similar extent (19.6%, Fig. 6D) as E329Q-NSF (16.3%, Fig. 4). In each case, the total amount of cellular $\alpha_5\beta_1$ was not altered (data not shown).

VAMP3 function is required for normal trafficking of $\alpha_5\beta_1$

The observations that both TeTx and E329Q-NSF reduced cell surface $\alpha_5\beta_1$ integrin levels without altering total cellular $\alpha_5\beta_1$ content suggest that $\alpha_5\beta_1$ is either accumulating within an intracellular compartment or being incorrectly trafficked to a compartment in which it normally does not reside. Previous studies have indicated that recycling of $\alpha_5\beta_1$ is regulated by a Rab11 compartment in some cell types [16,22] and we examined this possibility in CHO-K1 cells. Using a method similar to that described by Powelka et al. [22], antibody was applied externally to cells to monitor the subsequent endocytic trafficking of $\alpha_5\beta_1$ from the cell surface. It was found that an internalized population of this integrin accumulates in a compartment containing Rab11 (Fig. 7). After stimulation with serum for 10 min, $\alpha_5\beta_1$ was observed to be redistributed, having reduced overlap with Rab11 and accumulating at the perimeter of cells. The $\alpha_5\beta_1$/Rab11 compartment was not markedly altered in cells expressing TeTx; however, the serum-induced redistribution of $\alpha_5\beta_1$ to the cell perimeter was impaired by expression of TeTx (Fig. 7). A similar result was seen in cells expressing E329Q-NSF (data not shown). These results suggest that VAMP3 function is

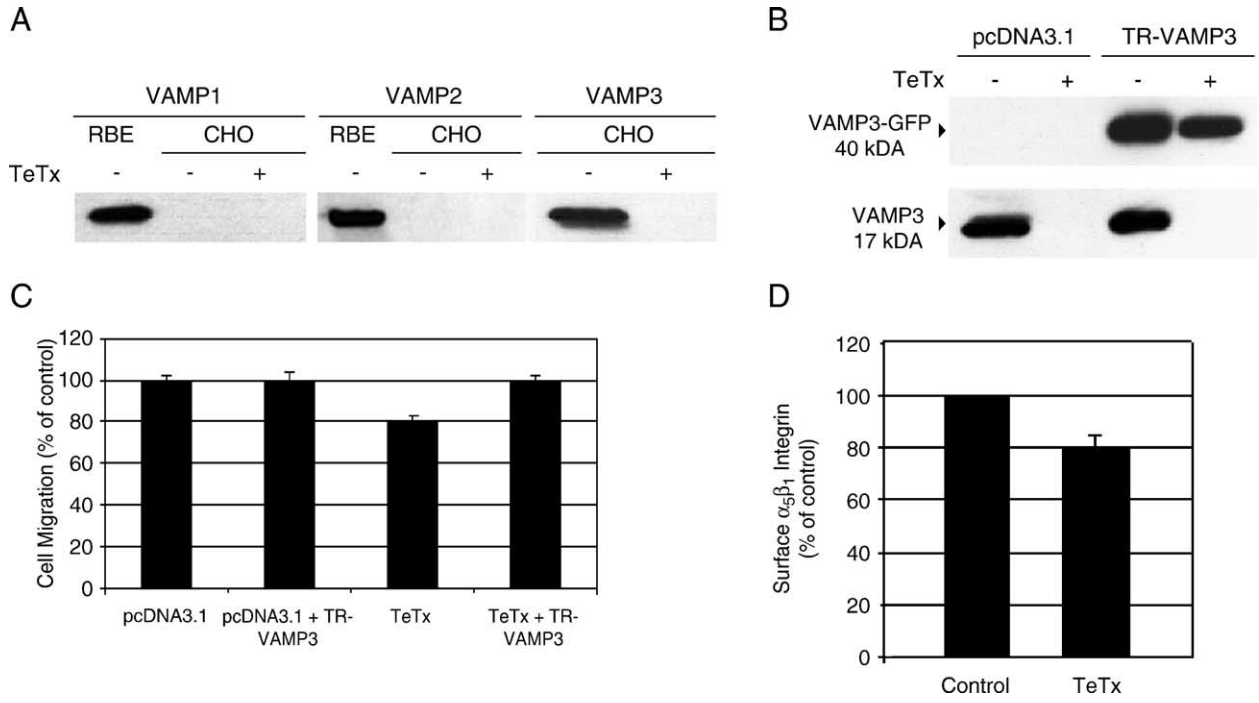

Fig. 6. Tetanus toxin impairs migration in CHO-K1 cells. (A) CHO-K1 cells were transfected with empty vector (pcDNA3.1) or Tetanus toxin-containing vector. Cells were lysed 24 h after transfection and extracted proteins were analysed by Western blotting with antibodies to VAMP1, VAMP2, or VAMP3. RBE is rat brain extract. (B) CHO-K1 cells were transiently co-transfected with empty vector (pcDNA3.1) or TeTx together with either GFP or GFP-tagged tetanus toxin-resistant VAMP3 (TR-VAMP3) at a 10:1 molar ratio. Twenty-four hours after transfection, cells were lysed and 30 μg of extracted protein per sample was analyzed by Western blotting with antibody to VAMP3. (C) CHO-K1 cells were co-transfected as in A. Twenty-four hours after transfection, cells were serum-starved for 3 h and cell migration assays were performed and quantified using GFP fluorescence as described before. Data are presented as the percentage of transfected cells migrated (normalized to pcDNA3.1-transfected control) and are the result of at least three independent experiments (*$P < 0.05$). (D) Cells were harvested and surface $\alpha_5\beta_1$ integrin was fluorescently labeled and quantified by flow cytometry. pcDNA3.1-transfected cells are represented by the control bar. Data are the result of at least three independent experiments and the samples are significantly different ($P < 0.01$).

required for the normal trafficking of $\alpha_5\beta_1$ during serum-induced cell migration.

Discussion

The results of this study show that SNARE-mediated membrane trafficking is required to support normal cell migration. Specifically, inhibition of the ATPase NSF by expression of a dominant-negative form of the enzyme (E329Q-NSF) impaired directed migration in CHO cells. E329Q-NSF-expressing cells were able to polarize normally in wound-healing assays, but were not able to extend lamellipodia at their leading edges. Importantly, cells expressing E329Q-NSF contained a similar number of focal adhesions as control counterparts, despite having a modestly reduced level of cell surface integrin. These findings are the first to directly implicate SNARE-mediated membrane traffic in the control of migration in mammalian cells. We conclude that SNARE-mediated membrane traffic regulates the intracellular distribution of integrin receptors and supports the membrane remodeling that is essential to lamellipodial extension.

SNARE activity is required for most, if not all, intracellular vesicle trafficking pathways, including pathways of exocytosis and secretion. Thus, our results, which clearly implicate SNARE function in regulating the intracellular distribution of integrins, are consistent with the notion that a SNARE-mediated vesicle trafficking pathway is required for the delivery of a subpopulation of integrins to the cell surface. When SNARE function was inhibited, there was a modest decrease in cell surface integrin and this correlated with a decrease in migration efficiency. This observation is consistent with a mathematical model of cell migration that posits that the efficiency of migration is determined by the following three factors: ligand concentration, integrin expression, and integrin-ligand affinity [38]. Given that the migration assays in the present study were conducted with a fibronectin concentration that was empirically determined to be optimal for the migration of CHO cells, it is reasonable that a decrease in cell surface integrin would impede migration on this substrate.

The results of experiments with TeTx indicate that VAMP3, a SNARE known to function in endocytic recycling and to mediate the fusion of vesicles at the plasma membrane [35,37], plays a role in regulating migration. It is interesting that inhibition of NSF decreased cell surface integrin expression to a similar degree as TeTx, but produced a larger decrease in cell migration (compare Figs. 1, 4 and 6D). These disproportionate effects indicate

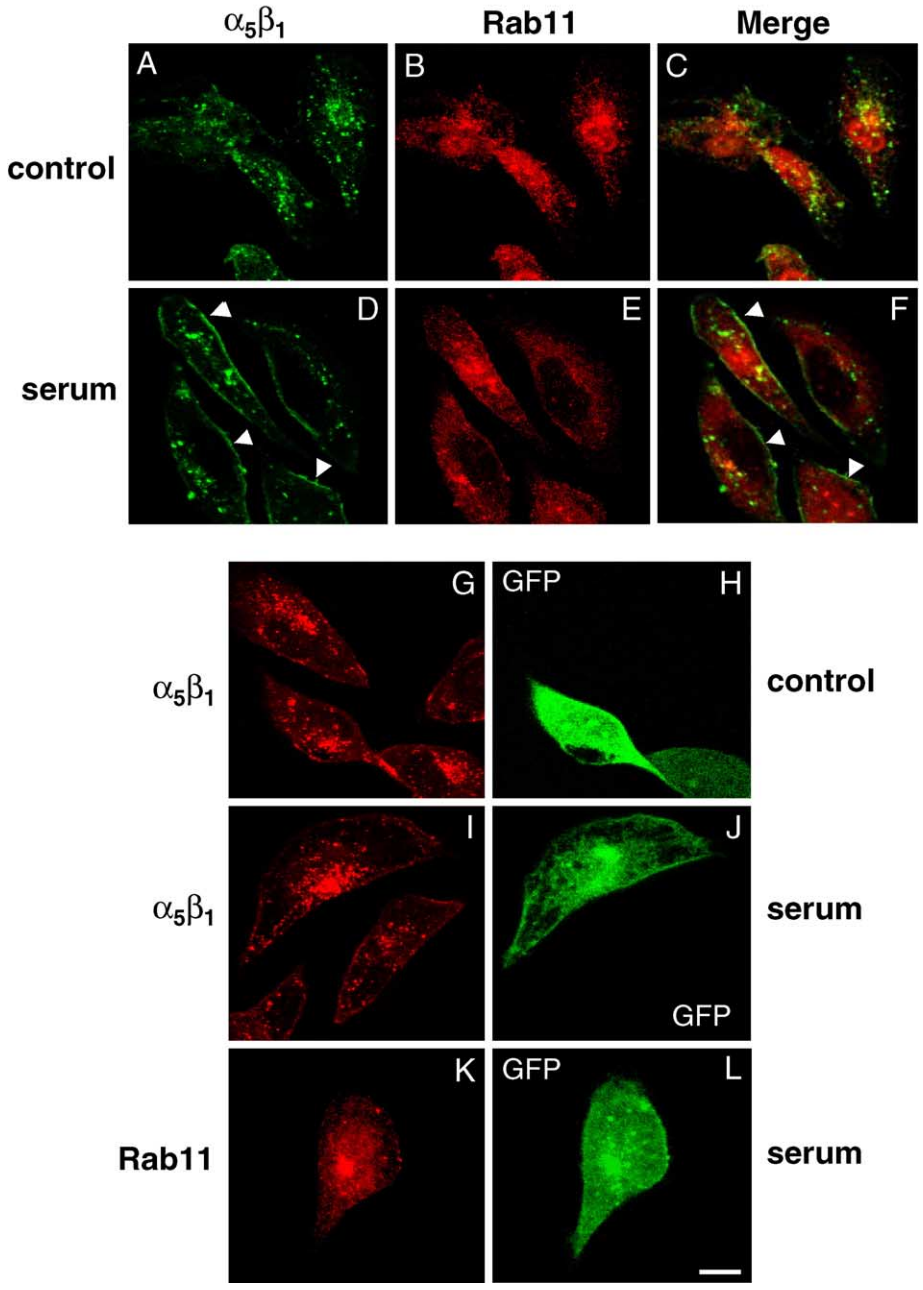

Fig. 7. Tetanus toxin perturbs trafficking of $\alpha_5\beta_1$ out of a Rab11 compartment. Serum-starved CHO-K1 cells were allowed to internalize anti-$\alpha_5\beta_1$ antibody by applying antibody externally and incubating at 37°C. Remaining cell surface antibody was then removed with trypsin. The samples were stimulated to mobilize integrins by addition of 10% serum for 10 min, then fixed, permeabilized and stained for the protein markers indicated. (A–C) Internalized $\alpha_5\beta_1$ (A) was imaged in cells counterstained for Rab11 (B). (C) Merged image of $\alpha_5\beta_1$ and Rab11. (D–F) Internalized $\alpha_5\beta_1$ (D) was imaged in cells counterstained for Rab11 (E) after stimulation with serum for 10 min. (F) Merged image of $\alpha_5\beta_1$ and Rab11. Arrowheads in D point to $\alpha_5\beta_1$ localized at cell perimeter. (G) $\alpha_5\beta_1$ compartment in samples (no serum) transfected with TeTx. (H) GFP signal in cells co-transfected with GFP and TeTx in panel G. (I) $\alpha_5\beta_1$ compartment in samples transfected with TeTx and stimulated with serum for 10 min. (J) GFP signal in cells co-transfected with GFP and TeTx in panel I. Note the difference between the $\alpha_5\beta_1$ compartment in the transfected serum-treated cell in I and the serum-treated cells in D. (K) Rab11 compartment in cell transfected with TeTx and stimulated with serum for 10 min. (L) GFP signal in cell co-transfected with GFP and TeTx in panel K. Scale bar = 10 μm.

that the defects in cell migration resulting from impaired SNARE function are due only in part to the attendant decreases in surface integrin. We speculate that SNARE-regulated vesicular traffic is necessary for the delivery of membrane and/or membrane components that support integrin function downstream of ligand binding. These results also suggest that only a subset of the SNARE-mediated membrane traffic pathways that support cell migration and that are regulated by NSF are mediated by VAMP3. While it is possible that in the absence of VAMP3 another tetanus toxin-insensitive SNARE can partially compensate for its function, it is also plausible that

membrane trafficking pathways are contributing to cell migration independently of the recycling compartment regulated by VAMP3.

We have identified a Rab11-containing endosomal compartment that is associated with the major pool of recycling $\alpha_5\beta_1$ integrin in CHO-K1 cells. Importantly, serum-induced trafficking of $\alpha_5\beta_1$ out of this compartment to the cell perimeter was impaired by treatment with either E329Q-NSF or TeTx. This indicates that SNARE function, including that of VAMP3, is required for normal trafficking of $\alpha_5\beta_1$ and suggests that perturbation of SNARE-regulated trafficking may contribute to the observed decreases of $\alpha_5\beta_1$ on the cell surface by altering the intracellular compartmentalization of the integrin. A role for Rab11 in the recycling of $\alpha_5\beta_1$ is consistent with results from other laboratories [16,22]. While the $\alpha_5\beta_1$ compartment studied in HeLa cells by Powelka et al. did not contain VAMP3, regulation of this compartment in the CHO cells used here is clearly dependent upon the function of this SNARE. It is therefore reasonable to propose that cell-type differences in the regulation of integrin recycling exist, possibly involving different SNARE proteins, and we are currently working to elucidate such differences.

Inhibition of either NSF activity or VAMP3 function produced similar effects on both the Rab11/$\alpha_5\beta_1$ compartment and on cell surface $\alpha_5\beta_1$ levels, yet blocking NSF activity caused a greater impairment in cell migration. This suggests that more than one SNARE-mediated membrane trafficking pathway is involved in the regulation of cell motility. It is predictable that all SNARE pathways would have been inhibited by E329Q-NSF and that, in the CHO cells used here, only VAMP3-regulated pathways would have been blocked by tetanus toxin. The notion that cell migration involves multiple intracellular trafficking pathways agrees with recent evidence indicating that integrin-mediated cell motility is dependent upon PKD-regulated traffic, both recycling and anterograde [19,20], as well as PKB/Akt-regulated traffic [21]. Additionally, integrin traffic appears to be regulated by Rab4 and Rab11 functioning in integrin-specific pathways [16,22]. It is now necessary to identify the SNARE proteins that mediate these specific pathways in different cell types.

The effects of blocking SNARE function on lamellipodial extension reported here are similar to the effects that such treatment has upon phagocytosis, a process which is mechanistically analogous to cell migration. During phagocytosis, it has been shown that inhibition of SNARE function, including expression of dominant-negative NSF, impairs the extension of membrane structures (pseudopodia) around the particle being ingested [27,39]. The formation of both lamellipodia and pseudopodia is thus partially dependent upon SNARE-mediated membrane traffic. Furthermore, like the extension of pseudopodia, lamellipodial protrusion requires actin polymerization to generate force on the membrane being remodeled, but we have not observed any changes in the morphology or function of the actin cytoskeleton in cells containing impaired SNARE function [27] (this study, data not shown). It is therefore likely that a direct effect of blocking SNARE function in migrating cells is an alteration to membrane remodeling at the leading edge.

The inability of cells deficient in SNARE function to form normal lamellipodia may be due in part to the observed reduction in cell surface integrin levels. For example, a deficit in a specific subpopulation of integrin receptors at the leading edge could result in a lack of firm adhesive contact between the membrane extension and the ECM substrate. This would mean that lamellipodia can be extended, but do not attach and are retracted. It is also possible that SNARE-regulated delivery of vesicles is required to provide the membrane with which the lamellipodial protrusion is formed. In this case, changes in membrane morphology at the leading edge might be minimal. Microscopic examination of lamellipodial protrusion in our system, using live cells, will help to discriminate between these two possibilities.

An additional explanation for our observations is that inhibition of SNARE-mediated membrane trafficking may interfere with the delivery of components required for the normal assembly and turnover of adhesive structures near the leading edge of the cell. In migrating cells, integrins in newly formed protrusions bind to the extracellular matrix and recruit proteins to their intracellular domains, ultimately forming focal adhesions. These adhesive structures include structural proteins such as paxillin, talin and vinculin, signal transducers such as FAK and ILK and the actin-nucleating Arp2/3 complex which helps modulate actin filament formation [40–43]. With adhesive sites forming at the leading edge of a migrating cell, it is reasonable to speculate that adhesion complex components may be specifically transported to that location. We have not detected any difference in the number of paxillin-containing focal adhesions between cells with impaired SNARE function and control cells; however, it remains a possibility that focal adhesion function and biochemical signaling have been altered. It is also important to note that the data presented here indicate that inhibition of SNARE-mediated membrane traffic did not completely block cell migration. Thus, there may be sufficient levels of the required adhesive/signaling components at the leading edge of cells to support a reduced level of cell motility in the absence of SNARE function. We are currently investigating this possibility by examining focal adhesion assembly in cells with impaired SNARE function.

In conclusion, this study has presented evidence of a role for SNARE-mediated membrane traffic in cell migration. Overall, the observations in the present study are consistent with the existing body of evidence suggesting that integrins and other cellular proteins are transported to the leading edge of migrating cells. As well, our studies build upon previous reports demonstrating that NSF function is required for cell locomotion in *D. discoideum* [44] and thus our results contribute to the growing understanding of

the central role for SNARE-mediated membrane traffic in eukaryotic cell function. Future studies will be directed at characterizing SNARE function in more detail to further elucidate the molecular mechanisms that control cell migration.

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