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Syntaxin 2 and Endobrevin Are Required for the Terminal Step of Cytokinesis in Mammalian Cells

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Summary

The terminal step of cytokinesis in animal cells is the abscission of the midbody, a cytoplasmic bridge that connects the two prospective daughter cells. Here we show that two members of the SNARE membrane fusion machinery, syntaxin 2 and endobrevin/VAMP-8, specifically localize to the midbody during cytokinesis in mammalian cells. Inhibition of their function by overexpression of nonmembrane-anchored mutants causes failure of cytokinesis leading to the formation of binucleated cells. Time-lapse microscopy shows that only midbody abscission but not further upstream events, such as furrowing, are affected. These results indicate that successful completion of cytokinesis requires a SNARE-mediated membrane fusion event and that this requirement is distinct from exocytic events that may be involved in prior ingression of the plasma membrane.

Introduction

Cytokinesis, the division of a cell into two daughter cells, is a fundamental process in biology common to all organisms (Field et al., 1999; Finger and White, 2002; Glotzer, 2001; O'Halloran, 2000; Zeitlin and Sullivan, 2001). In animal cells, cytokinesis is a multistep process that involves the assembly of an actin/myosin-dependent contractile ring that guides the invagination of the plasma membrane leading to the formation of a cleavage furrow. Furrowing proceeds until the cytoplasm is constricted to a narrow bridge—termed the midbody—that contains the remnants of the spindle microtubules and connects the two prospective daughter cells. The terminal step of cytokinesis is the abscission of the midbody, which leads to completely separate daughter cells. The mechanism by which midbody abscission is achieved has remained unknown. However, this step would be expected to involve membrane fusion events. Otherwise, the plasma membranes of the newly generated daughter cells would be ruptured.

So far, requirements for membrane fusion events during cytokinesis have been identified only for stages that precede midbody abscission. For example, exocytosis is required to supply the necessary additional surface area required for furrow ingression during cytokinesis in *C. elegans* and sea urchin embryos (Jantsch-Plunger and Glotzer, 1999; Shuster and Burgess, 2002). In *C. elegans*, furrow ingression appears to require Syn-4, a syntaxin family member of the SNARE membrane fusion machinery (Jantsch-Plunger and Glotzer, 1999). The function of Syn-4 may not be restricted to furrow ingression, however, because its disruption also causes defects in nuclear envelope reformation. Rab3, a member of a family of proteins implicated in the regulation of SNARE function, has been implicated in furrow ingression in sea urchin embryos, but it may also act at earlier stages because its disruption also leads to failure of nuclear division (Conner and Wessel, 2000). SNARE proteins can also be involved in animal cytokinesis in an indirect fashion. For example, a syntaxin has been implicated previously in cell division in sea urchin embryos. Functional disruption of this syntaxin affects nuclear division, which subsequently appears to inhibit cytokinesis indirectly due to a halt of cell cycle progression (Conner and Wessel, 1999). The mechanism of cytokinesis in plant cells differs from that in animal cells in that no furrowing of the plasma membrane occurs, and no midbody-like bridge is formed. Instead, plant cells assemble a new plasma membrane and cell wall—termed the cell plate—in the middle of the dividing cells. The cell plate is assembled by the fusion of small vesicles with each other and with the growing cell plate in a process that requires the action of members of the SNARE membrane fusion machinery (Assaad et al., 2001; Heese et al., 2001; Lauber et al., 1997; Waizenegger et al., 2000).

While these studies have established that membrane fusion events are required for steps during cytokinesis prior to midbody abscission, the mechanism of this terminal step has remained unknown. Interestingly, recent experiments in *C. elegans* have indicated that midbody abscission can be inhibited using the fungal metabolite brefeldin A—a drug that disrupts several organelles and trafficking pathways—under conditions that do not affect furrow ingression (Skop et al., 2001). This suggested that different cellular machineries may control fusion events that facilitate furrow ingression and midbody abscission.

Here we report the identification of two members of the SNARE membrane fusion machinery, syntaxin 2 and endobrevin/VAMP-8, which specifically localize to the midbody region during cytokinesis in mammalian cells. Their functional inhibition causes failure of midbody abscission while earlier steps of cytokinesis are unaffected. These results indicate that the terminal step of cytokinesis is not a passive "ripping-apart" or "pinching-off" mechanism but is regulated by a SNARE-mediated membrane fusion event that is distinct from exocytic events that are involved in prior ingression of the plasma membrane.

Results and Discussion

Syntaxin 2 Localizes to the Midbody

Membrane fusion events in intracellular vesicle trafficking pathways are generally mediated by proteins of the

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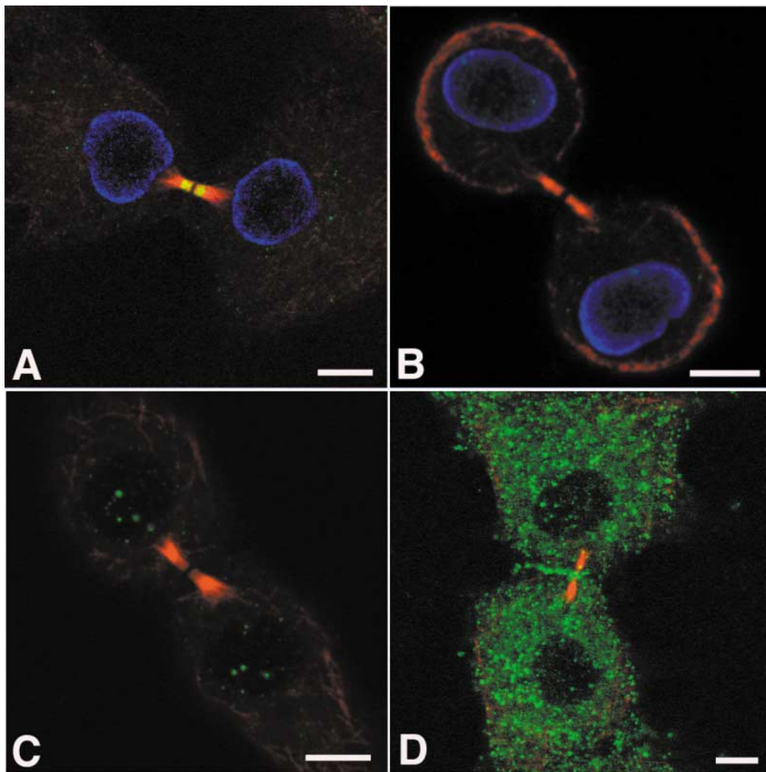


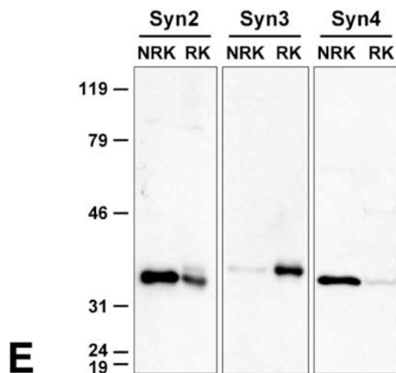


Figure 1. Syntaxin 2 Localizes to the Midbody during Cytokinesis

(A) Coimmunolocalization of syntaxin 2 (green) and β-tubulin (red) in NRK cells during the midbody stage of cytokinesis. Nuclei are stained with DAPI (blue).

(B) Competition with purified antigen eliminates syntaxin 2-specific staining.

(C and D) Neither syntaxin 3 (C) nor syntaxin 4 (D) localize to the midbody of dividing cells. Scale bars, 5 μm.

(E) Immunoblot analysis of NRK cell lysates shows that syntaxins 2 and 4 are abundantly expressed in NRK cells whereas the expression level of syntaxin 3 is relatively low. Equal amounts (15 μg protein) of total rat kidney (RK) lysates were used as controls. Molecular weight makers are indicated in kilodaltons.

SNARE superfamily, which consists of several subfamilies, including syntaxins (Chen and Scheller, 2001; Jahn and Sudhof, 1999; Weimbs et al., 1997). Our analysis of the localization of syntaxin 2 in cultured proliferating NRK (normal rat kidney) cells led to the following serendipitous finding. An affinity-purified antibody against syntaxin 2 strongly labeled small structures in a fraction of the cell population. Colabeling for β-tubulin identified these structures as midbodies. Syntaxin 2 immunoreactivity localized to distinct regions of $\sim$1 μm apparent diameter on either side of the midbody (Figure 1A). These syntaxin 2 regions were intersected by microtubules. This staining pattern was consistently observed using two independently raised syntaxin 2 antibodies and could be eliminated by competing antigen, indicating that it is specific (Figure 1B). Identical staining patterns were also observed with several other mammalian cell lines including HEK293 and CHO cells (data not shown). It should be noted that a well-known apparent gap exists in the center of the midzone in which tightly packed matrix proteins prevent antibody staining to microtubules even though they are present (Mullins and McIntosh, 1982). By analogy, it is therefore possible that the localization of syntaxin 2 may extend further toward the midzone than can be demonstrated by immunostaining. In interphase cells, syntaxin 2 localized to the plasma membrane and intracellular vesicles (data not shown) as previously reported (Quinones et al., 1999).

Syntaxin 2 is a ubiquitously expressed t-SNARE (Bennett et al., 1993) that has been reported to be targeted to the plasma membrane in several cell types, including polarized Madin Darby canine kidney (MDCK) cells (Li et al., 2002; Low et al., 1996, 2000). Two other widely expressed plasma membrane t-SNAREs are syntaxins 3 and 4 (Li et al., 2002; Low et al., 1996). Western blot analysis showed that NRK cells express all three syntaxins (Figure 1E). However, neither syntaxin 3 nor syntaxin 4 exhibited the same midbody localization as syntaxin 2 during cytokinesis (Figures 1C and 1D).

The subcellular steady-state location of a given t-SNARE

generally corresponds to the site at which this t-SNARE functions. The localization of syntaxin 2 at the midbody therefore suggested that it may be involved in a fusion event required for cytokinesis. We considered two possibilities. First, syntaxin 2 may be involved in increasing the cell surface area during furrowing by mediating the fusion of vesicles with the plasma membrane close to the site of ingression. Second, syntaxin 2 may be directly involved in the final abscission of the midbody to result in completely separated daughter cells.

Syntaxin 2 Function Is Required for Cytokinesis

To investigate whether syntaxin 2 function is required for cytokinesis and to examine its mechanism of action, we employed a dominant-negative approach. The overall domain structure of syntaxins is highly conserved (Weimbs et al., 1997, 1998), and they are characterized by a C-terminal transmembrane anchor, while the rest of the molecule protrudes into the cytoplasm. Recombinant soluble SNAREs that lack the membrane anchors are known to inhibit membrane fusion by forming nonfunctional complexes with endogenous SNARE proteins (Hua and Scheller, 2001). A brain-specific, alternatively spliced isoform of syntaxin 2, termed syntaxin 2D, has previously been identified that lacks a transmembrane anchor, while the remainder of the cytoplasmic domain is identical to full-length syntaxin 2 (Quinones et al., 1999). The function of syntaxin 2D is unknown. However, it was reported to be a soluble cytoplasmic protein (Quinones et al., 1999) and would be predicted to act as a dominant-negative inhibitor of the function of membrane-anchored syntaxin 2.

We expressed syntaxin 2D in MDCK cells using an adenovirus vector with a tetracycline-regulatable promotor. Immunofluorescence analysis confirmed the cytoplasmic localization of syntaxin 2D (Figure 2A). Syntaxin 2D expression for 16 hr resulted in a high frequency of binucleated cells, indicating that the cells had undergone nuclear division in the absence of cytokinesis (Figures 2A and 2D). This effect could be prevented by suppressing syntaxin 2D expression by the addition of doxycycline, indicating that the observed block of cytokinesis is not due to the adenoviral infection. Furthermore, adenovirus-mediated expression of the membrane-anchored, full-length syntaxin 2A did not result in an increase in binucleated cells (Figure 2D). Given that only $\sim$50% of the cells underwent mitosis during the course of these experiments, we estimate that cytokinesis failed in approximately 60% of the mitotic events in cells that expressed syntaxin 2D. These results indicate that syntaxin 2 function is required for cytokinesis.

Syntaxin 2 Function Is Required for Midbody Abscission

To distinguish whether syntaxin 2 inhibition prevents the ingression of the cleavage furrow or the abscission of the midbody, syntaxin 2D-expressing cells were investigated by time-lapse phase-contrast microscopy (see Supplemental Data, Movie S1 [http://www.developmentalcell.com/cgi/content/full/4/5/753/DC1]). Figure 2C shows representative frames. In six independent time-lapse experiments, 41 failed cytokinesis events were observed that resulted in the formation of binucleated cells. In all cases, nuclear division, cleavage furrow formation and ingression, and the formation of midbodies were indistinguishable from controls. However, the cells were unable to undergo midbody abscission. The average time that the syntaxin 2D-expressing cells remained in the midbody stage was 153 min (range 64–355 min, n = 41), after which midbody regression occurred to lead to binucleated cells.

In two additional independent time-lapse experiments, the fate of the cell population was quantified on a cell-by-cell basis. While approximately 60% of cells did not undergo mitosis during the recordings (14 hr), of those that did, 48 failed in cytokinesis (53%) and 43 completed it successfully (47%). The heterogeneity of expression levels of syntaxin 2D in the cell population and the fact that the expression level necessarily has to ramp up during the course of the experiment are likely reasons for the observed inhibition of cytokinesis in less than 100% of the cells. Furthermore, this suggests that a minimum threshold level of cellular syntaxin 2D is required for successful inhibition, compatible with a dominant-negative effect.

As a further control for the specificity of the dominant-negative inhibition of syntaxin 2 function, truncated versions of syntaxins 3 and 4—lacking the transmembrane anchors—were expressed in MDCK cells by transient transfection. This was compared to transient transfection of syntaxin 2A or 2D cDNAs. All cDNAs were inserted into the identical plasmid vector, and side-by-side transient expression resulted in comparable levels of expressed proteins. Similar to the adenoviral gene transfer above, expression of syntaxin 2D for 24 hr resulted in a high frequency of binucleated cells (Figure 2E). In contrast, neither expression of the membrane-anchored syntaxin 2A nor of the truncated syntaxins 3 or 4 had this effect. This result indicates that the dominant-negative inhibition by nonmembrane-anchored syntaxins is specific and that syntaxin 2 is specifically involved in cytokinesis.

To investigate whether syntaxin 2 inhibition might affect the reassembly of the nuclear envelope, the binucleated cells were immunostained with antibodies against the nuclear transport factor p97 (Figure 2B) or lamin B_2 (data not shown). The nuclear envelopes of the binucleated cells appeared to be complete and intact and were indistinguishable from those of control cells, indicating that syntaxin 2 inhibition has no effect on the nuclear envelope and that nuclear division was unperturbed. We also did not observe evidence for micronuclei or nuclear buds in the binucleated cells after syntaxin 2 inhibition. These defects would be indicators of loss or malsegregation of chromosomes as a result of defects in the spindle or centromeres or as a consequence of chromosome undercondensation (Fenech and Crott, 2002). Overall, these results indicate that syntaxin 2 is required specifically for midbody abscission but not for further upstream events of mitosis such as chromosome segregation, nuclear envelope reassembly, furrowing, etc. Furthermore, the results indicate for the first time that midbody abscission involves a SNARE-mediated fusion event and suggest that this event utilizes a fusion machinery that differs from that which is required for exocytosis for the delivery of new membrane to aid in furrow ingression. Finally, if midbody abscission is blocked by syntaxin 2 inhibition, cells cannot otherwise “rip apart” or “pinch off” to complete cytokinesis.

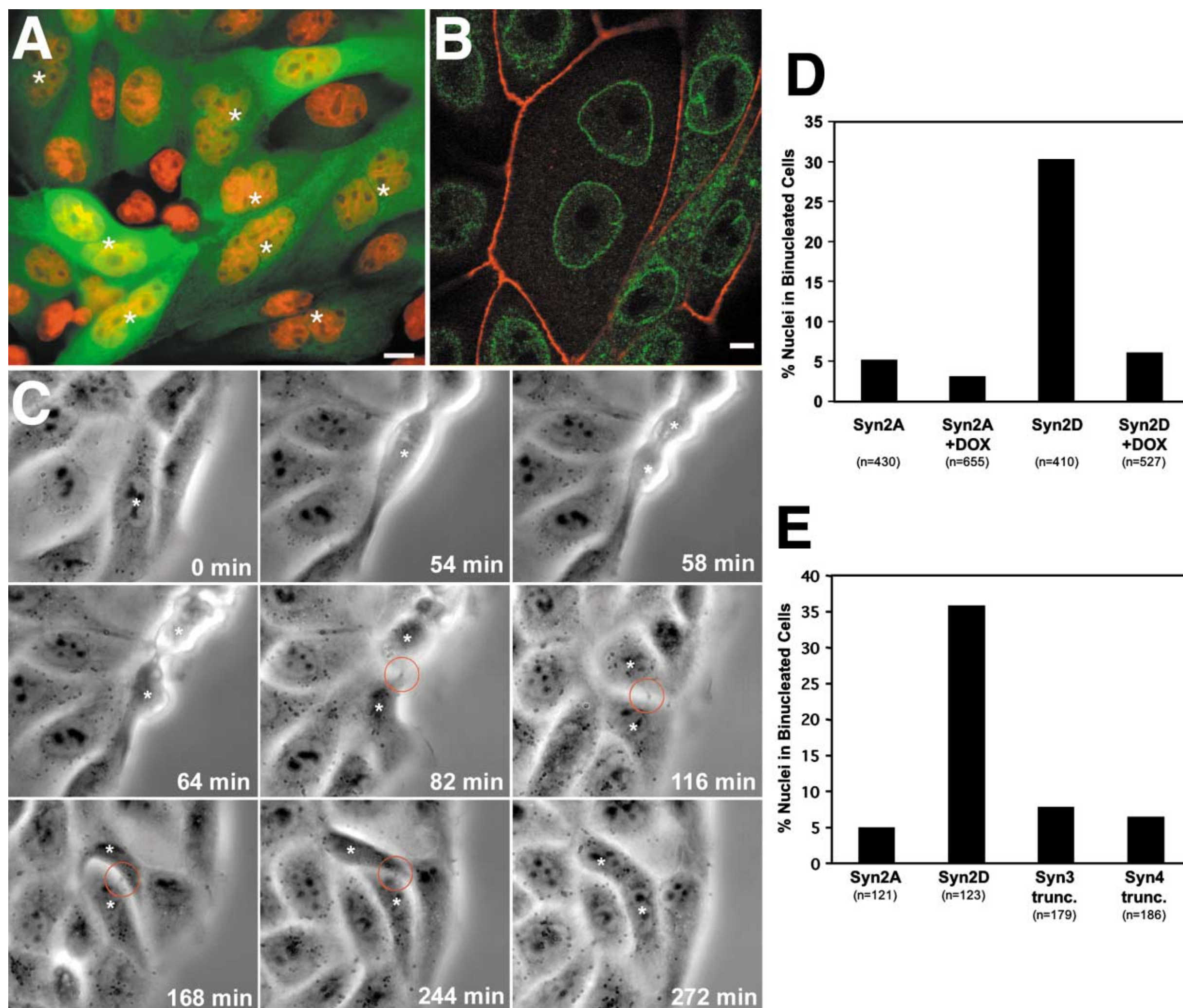


Figure 2. Expression of Soluble Syntaxin 2 Inhibits Midbody Abscission Resulting in Binucleated Cells

(A) Syntaxin 2D, a splice isoform lacking a transmembrane anchor, was expressed in MDCK cells. Immunostaining for syntaxin 2 (green) reveals expressing cells. Binucleated cells are denoted by asterisks. Scale bar, 5 μm.

(B) Binucleated cells formed after 16 hr expression of syntaxin 2D were subjected to double immunostaining for the nuclear transport factor p97 (green) and the tight junction protein ZO-1 (red). Note that the nuclei of binucleated cells exhibited normal p97 staining, indicating that nuclear division and reformation of the nuclear envelopes was unaffected.

(C) Frames of time-lapse phase contrast microscopy of MDCK cells expressing syntaxin 2D. For orientation, the cell if interest is highlighted by asterisks and the midbody is circled (see Supplemental Data, Movie S1 [http://www.developmentalcell.com/cgi/content/full/4/5/753/DC1]).

(D) Quantification of failed cytokinesis after 16 hr expression of the full-length syntaxin 2A or the truncated syntaxin 2D using adenovirus vectors calculated as the fraction of nuclei in binucleated cells as the percentage of the total nuclei. As negative controls, syntaxin expression was prevented by the addition of doxycycline (+DOX). The total numbers of nuclei counted for each condition are indicated.

(E) Failed cytokinesis after 24 hr expression of syntaxin 2A, syntaxin 2D, or truncated versions of syntaxins 3 or 4 lacking transmembrane anchors by plasmid-mediated transient transfection.

Endobrevin/VAMP-8 Functions Together with Syntaxin 2 during Midbody Abscission

If syntaxin 2 mediates a membrane fusion event that is required for the severing of the midbody, it would be predicted to involve other members of the SNARE machinery as well. In other intracellular fusion events, small v-SNAREs of the synaptobrevin/VAMP family mediate membrane fusion in concert with syntaxins. A v-SNARE involved in cytokinesis would be expected to exhibit a relatively ubiquitous tissue expression pattern. We investigated whether the two ubiquitously expressed v-SNAREs cellubrevin/VAMP-3 or endobrevin/VAMP-8 localize to the midbody region during cytokinesis. Figure 3A shows that cellubrevin is only found on intracellular vesicles but not at the midbody during cytokinesis. In contrast, endobrevin is highly concentrated at the midbody in a staining pattern very similar to that of syntaxin 2 (Figures 3B and 3D). Again, the immunosignal could be eliminated by competition with antigen (Figure 3C), and two independent endobrevin antibodies resulted in identical staining patterns (data not shown). Double immunofluorescence microscopy with antibodies against syntaxin 2 and endobrevin revealed nearly completely overlapping localizations (Figure 3E). During interphase,

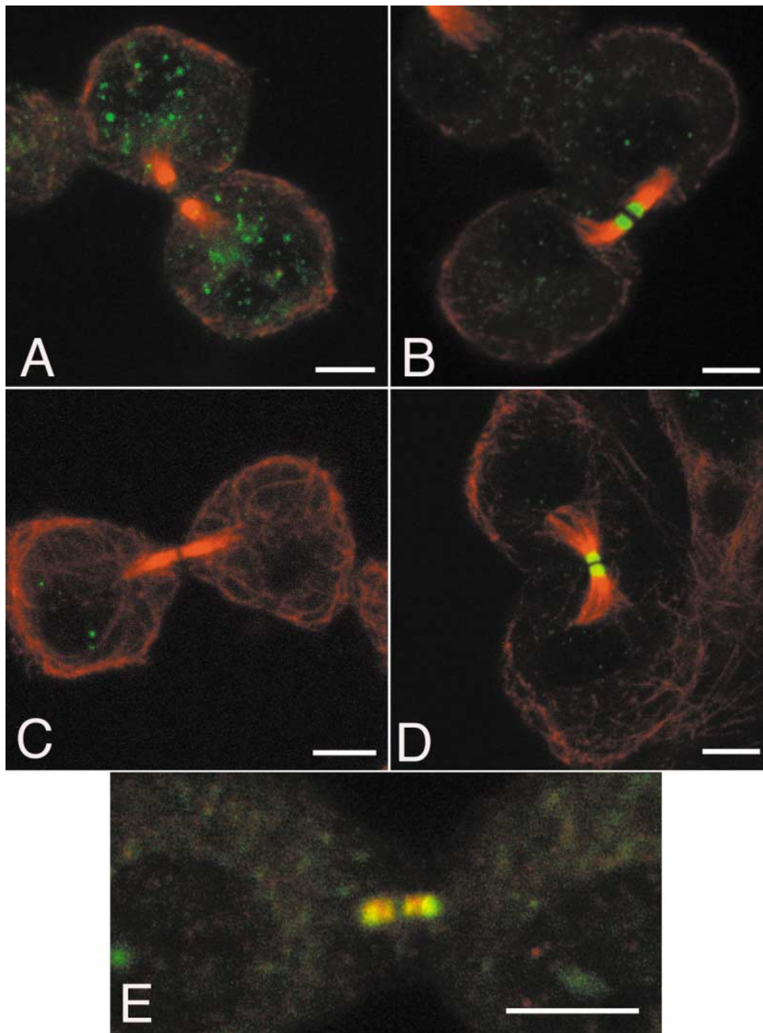


Figure 3. Endobrevin Colocalizes with Syntaxin 2 on the Midbody

(A) Cellubrevin (green) localizes to intracellular vesicles in late telophase NRK cells but not to the midbody, which is identified by immunostaining for β-tubulin (red).

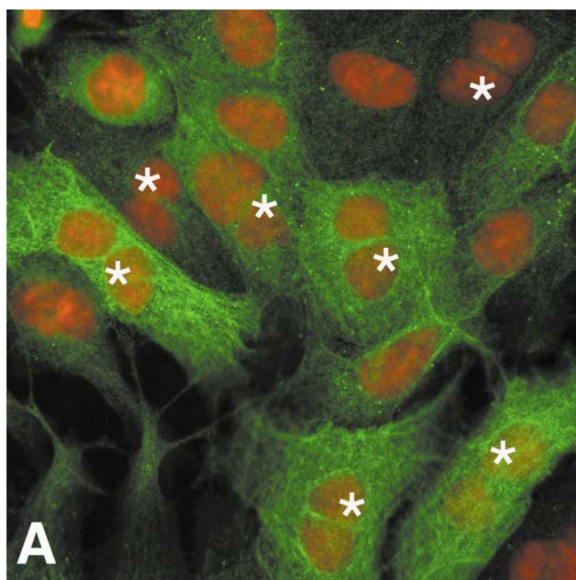
(B–D) Endobrevin (green) localizes to midbody in NRK cells (B). The immunostaining for endobrevin is eliminated by competition with total bacterial lysate containing GST-endobrevin (C) but not by lysate containing GST (D).

(E) Coimmunostaining for syntaxin 2 (green) and endobrevin (red) reveals their colocalization on the midbody of NRK cells. Scale bars, 5 μm.

endobrevin localizes to several intracellular organelles, including tubulovesicular structures, small vesicles, multivesicular bodies, vacuolar endosomes, TGN, plasma membrane, and clathrin-coated pits in NRK cells and has been reported to mediate homotypic fusion of early endosomes and late endosomes (Antonin et al., 2000).

To investigate whether endobrevin is functionally involved in cytokinesis, we constructed a truncation mutant lacking the C-terminal transmembrane anchor and expressed it using a tetracycline-regulatable adenoviral vector as described above for syntaxin 2D. Figure 4A shows that expressed truncated endobrevin distributes throughout the cytoplasm and results in a high percentage of binucleated cells after 16 hr. As a control, when the expression of truncated endobrevin was prevented by the inclusion of doxycycline the formation of binucleated cells was suppressed (Figure 4B). These results indicate that truncated endobrevin acts as a dominant-negative inhibitor of membrane-anchored endobrevin resulting in inhibition of cytokinesis. Time-lapse microscopy revealed that endobrevin inhibition did not interfere with events upstream of midbody-formation but resulted in the inability to cleave the midbodies (see Supplemental Data, Movie S2 [http://www.developmentalcell.com/cgi/content/full/4/5/753/DC1]). The average time between midbody formation and regression into binucleated cells was 173 min (range 80–345 min, $n = 45$). This phenotype was indistinguishable from that of syntaxin 2 inhibition as described above, suggesting that syntaxin 2 and endobrevin act together at the same step during midbody abscission.

Collectively, these results show for the first time that midbody abscission requires the action of members of the SNARE membrane fusion machinery. Inhibition of syntaxin 2 or endobrevin had no apparent effect on cleavage furrow invagination, nuclear division, reformation of the nuclear envelope, or other mitotic events. It is therefore unlikely that these SNAREs are involved in any mitotic step prior to midbody abscission. Since cleavage furrow invagination is believed to require exocytosis for the insertion of additional plasma membrane, it is likely that other SNAREs are involved in this process in mammalian cells. A possible candidate is syntaxin 4, which we found to localize to the ingressed plasma membranes separating the prospective daughter cells prior to midbody abscission (see Figure 1D). This would be analogous to the proposed function of SYN-4 in *C. elegans* (Jantsch-Plunger and Glotzer, 1999). Note that



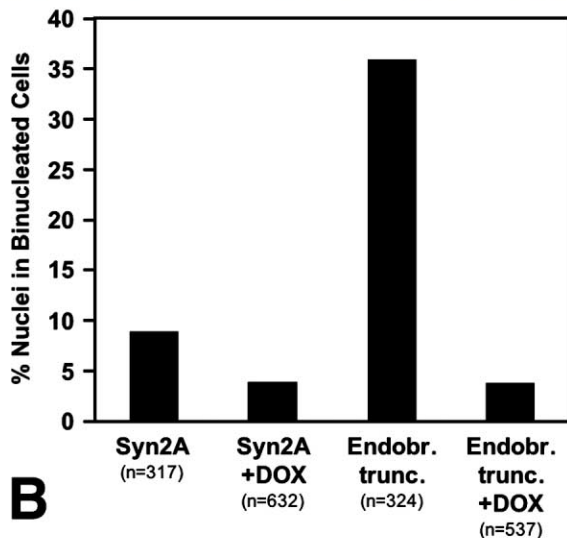


Figure 4. Endobrevin Function Is Required for Cytokinesis

(A) Expression of truncated endobrevin lacking its transmembrane anchor (green) in MDCK cells for 16 hr results in the formation of binucleated cells (denoted by asterisks).

(B) Quantification of failed cytokinesis after expression of truncated endobrevin or syntaxin 2A as a control for 16 hr. As a further control, expression was suppressed by doxycycline (DOX).

mammalian and *C. elegans* SNAREs are too divergent to allow assignment of orthologs by sequence comparison (Jantsch-Plunger and Glotzer, 1999; Weimbs et al., 1997). Therefore, despite the coincidental similarity of their names, it remains to be established whether the mammalian syntaxin 4 may have the equivalent role of the *C. elegans* SYN-4 in cleavage furrow ingression.

Most proteins involved in cytokinesis also have other functions in nondividing cells. This is likely the case for syntaxin 2 and endobrevin as well. Both are expressed in nondividing cells such as renal epithelial cells (Li et al., 2002) and the retinal pigment epithelium (Low et al., 2002). Syntaxin 2 is has previously been implicated in the sperm acrosome reaction (Katafuchi et al., 2000) as well as zymogen granule exocytosis in pancreatic acinar cells (Hansen et al., 1999), and endobrevin has been reported to be involved in endosome fusion (Antonin et al., 2000). Our finding that syntaxin 2 and endobrevin function in midbody abscission during cell division indicates that this terminal step of cytokinesis utilizes a SNARE machinery that is distinct from those involved in prior mitotic steps that require membrane fusion such as furrowing. If the function of syntaxin 2 or endobrevin is inhibited, cell division can not be completed, indicating that other SNAREs can not substitute their function. This suggests that midbody abscission is a highly regulated, active process, and that mammalian cells possess no alternative mechanisms that can accomplish the breakage of this narrow bridge.

Cell division is not only a fundamental biological process but is also of particular interest as a target for antitumor strategies. Currently used anti-tumor compounds target the cell cycle at various steps. The identification of molecules involved in the terminal step of cytokinesis may provide potential new targets that may be exploited for cancer therapy.

Experimental Procedures

Antibodies

Affinity-purified antibodies against the cytoplasmic domains of rat syntaxins 2, 3, and 4 have been described previously (Low et al., 2000). An antibody against the cytoplasmic domain of endobrevin was raised and affinity purified equivalently as described previously (Li et al., 2002). As confirmatory controls, independently raised affinity-purified antibodies against syntaxin 2 (Quinones et al., 1999) and endobrevin (gift from Wanjin Hong, IMCB, Singapore) were used. A monoclonal β-tubulin antibody developed by Michael Klymkowsky was obtained from the Developmental Studies Hybridoma Bank, The University of Iowa. Antibodies against the Nuclear Transport Factor p97 and ZO-1 were from ABR (Golden, CO) and Chemicon (Temecula, CA), respectively.

Cell Culture and Immunolocalization

NRK cells (from ATCC) were cultured in DMEM with sodium pyruvate, 10% FBS and penicillin and streptomycin. MDCK cells were cultured as described (Low et al., 2000). Cells were fixed in methanol and subjected to immunostaining and confocal fluorescence microscopy as described previously (Low et al., 2000). For localizing simultaneously two proteins recognized by rabbit primary antibodies (syntaxin 2 and endobrevin), fluorescein-labeled F_{ab} fragments of the secondary antibody (Jackson ImmunoResearch, West Grove, PA) were used after incubation with the first rabbit primary antibody. The cells were briefly fixed again with 4% paraformaldehyde and then incubated with the second rabbit primary antibody, followed by Texas red-labeled secondary antibody (Weimbs et al., 2003). Antibody concentrations were titered so that all negative controls were negative.

Expression of SNARE Cytoplasmic Domains

The adenovirus vectors for tetracycline-regulated expression of rat syntaxins 2A and 2D have been described previously (Quinones et al., 1999). The identical vector system was used for the expression of truncated endobrevin lacking its transmembrane domain. MDCK cells stably expressing the TET transactivator (Clontech, Palo Alto, CA) were infected with virus numbers titered to result in ~80%–90% of expressing cells after 16 hr. After fixation, double immunostaining for the respective truncated SNARE and the p58 endogenous plasma membrane marker (Low et al., 2000), and nuclear staining with DAPI, random fields were imaged by fluorescence microscopy, and the number of nuclei in mono- and binucleated cells were counted manually. The fraction of nuclei in binucleated cells as a percentage of the total nuclei was expressed as a measure for the failure in cytokinesis. For plasmid-mediated transient transfection

experiments, the cDNAs encoding syntaxin 2A or 2D or truncated versions of syntaxin 3 or 4 were inserted into the vector pcDNA4/TO and transfected into MDCK cells cultured on glass cover slips using the ExGen 500 transfection reagent (Fermentas, Hanover, MD). This resulted in comparable expression levels as assessed by fluorescence microscopy. After 24 hr, analysis of failed cytokinesis was carried out as described above.

Time-Lapse Microscopy

Truncated SNAREs were expressed in MDCK cells as described above. Approximately 8 hr postinfection, cells were subjected to time-lapse phase contrast microscopy (2 minutes/frame) using a fully motorized Leica DMIRB microscope equipped with a temperature-, CO_2-, and humidity-controlled environmental chamber. Images were processed using Metamorph, Adobe Photoshop, and QuickTime.

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