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**Tetanus neurotoxin-mediated cleavage of cellubrevin impairs epithelial
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migration and integrin-dependent cell adhesion**

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Abstract

A role for endocytosis and exocytosis in cell migration has been proposed but not yet demonstrated. Here we show that cellubrevin, an early endosomal v-SNARE, mediates trafficking in the lamellipod of migrating epithelial cells and partially colocalizes with markers of focal contacts. Expression of tetanus neurotoxin, which selectively cleaves cellubrevin, significantly reduced the speed of migrating epithelial cells. Furthermore, expression of tetanus neurotoxin enhanced the adhesion of epithelial cells to collagen, laminin, fibronectin and E-cadherin, altered spreading on collagen, and impaired the recycling of $\beta 1$ integrins. These results suggest that cellubrevin-dependent membrane trafficking participates in cell motility through the regulation of cell adhesion.

Running Title: Cellubrevin and cell migration

Introduction

Cell migration and cell adhesion are two interdependent, well-conserved mechanisms of foremost importance during embryonic development, and are both pathologically altered in metastatic cancer cells (1) (2). In order for a cell to migrate, it first develops a polarized phenotype with a lamellipod at the front of the cell, whereby the migration front leads the movement, attaching to the substrate, and propelling the rest of the cell forward. Cell migration can be reconstituted *in vitro* by treating cells with motogenic stimuli such as Hepatocyte Growth Factor (HGF, also called scatter factor) (3), or by mechanically wounding a monolayer of epithelial cells, such as Madin Darby Canine Kidney (MDCK), stimulating cell migration on each side of the wound towards the opposite side, thereby closing the gap (4).

Two non-exclusive models have been proposed to explain the progression of the migration front of motile cells. The first model is based on a large body of evidence from studies in mono- as well as multi-cellular eukaryotes that demonstrates a fundamental role for the cytoskeleton in the migration front. Dynamic reorganization of the actin cytoskeleton is controlled by the small GTPases of the Rho subfamily (5, 6) and these GTPases, as well as actin and tubulin, play a major role in cell motility (7). A second model has emerged that proposes a role for exocytosis and endocytosis in cell motility. Endocytosis and exocytosis occur in the lamellipods of migrating cells (8-10) and could contribute to the extension of the cell border (11) and/or could bring receptors necessary for the binding of chemotactic ligands and for cell attachment to substrates (12, 13).

Cellubrevin (Cb) is a vesicular Soluble N-ethylmaleimide sensitive factor Attachment protein Receptor (v-SNARE), homologous to the neuronal synaptobrevins 1/2 (Syb), and is also a substrate of tetanus neurotoxin (TeNT) (14). Syb and Cb are the only known substrates of TeNT and TeNT is considered to be among the most selective toxins selected by pathological prokaryotes during evolution (15, 16). The function of Cb has been explored by treating cells with TeNT and by homologous recombination. The first approach demonstrated an important function of Cb in the recycling of plasma membrane receptors including transferrin receptor (17) and T cell receptors to the immunological synapse (18). In addition, Cb is involved in pathways implicating early endosomes such as apical transport of H⁺-ATPase (19), focal exocytosis at sites of phagocytosis (20, 21), release of retroviruses that assemble in endosomes (22), and the endosome to trans-Golgi network retrograde transport of Shiga toxin (23). In contrast, Cb knock-

out mice showed a lack of phenotype (24-26) that could result from functional redundancy with Syb1/2, endobrevin/VAMP8, TI-VAMP/VAMP7 or yet another v- SNARE that acute TeNT treatment may circumvent. In this paper, we set out to understand the role of Cb in cell migration and adhesion by testing the effect of TeNT in the epithelial MDCK cell line.

Materials and Methods

Antibodies and DNA Constructs

Mouse monoclonal antibodies anti-Green Fluorescent Protein GFP (clone 7.1 and 13.1) was obtained from Roche Diagnostics (Indianapolis, IN); anti-Talin (clone 8d4) from Sigma (St Louis, MI, USA); anti FAK (clone 4.47) from Upstate Biotechnology (NY) and anti-Phospho-Tyrosine (P-Tyr-100) from Cell Signaling Technology (Beverly, MA). Monoclonal antibodies against TeNT and brevins (Cl10.1) were generous gifts of Drs H. Niemann (Hannover Medical School, FRG), and R. Jahn (MPI, Gottingen, FRG) respectively. The mouse monoclonal antibody anti- β 1 integrin (Cl18) from Transduction Laboratories (Lexington, KY) was used for Western Blotting and the purified hamster monoclonal antibody (clone Ha2/5) for anti- β 1 integrin antibody uptake experiments (BD bioscience, Bedford, MA). Alexa Fluor488- and Alexa Fluor 568-coupled phalloidin was from Molecular Probes (Eugene, OR, USA). GFP-Cb stably expressing MDCK cells have been described previously (27). Wild-type and E234Q inactive TeNT in pCMV were described previously (14). The GFP-tagged VW Cb mutant, resistant to the proteolytic action of TeNT (GFPCb VW) was kindly provided by Dr. R. Regazzi (Univ. Lausanne, Switzerland) (28).

Cell Culture and Transfection

MDCK cells were cultured in DMEM with 7% FCS and transfected by electroporation. MDCK cells expressing GFP-Cb were cotransfected with a pCMV vector expressing either wild-type or E234Q inactive light chains of TeNT and a pPur vector conferring puromycin resistance (Clontech, Palo Alto, CA, USA). Cells were selected in medium containing 200 μ g/ml G418 and 4 μ g/ml Puromycin.

For HGF treatment, cells were cultured 16 to 24 hours in DMEM with 1% FCS supplemented by 20 ng/ml human HGF recombinant (Calbiochem, San Diego, Ca, USA)

Treatment of cell extracts with neurotoxins

CaCo-2, PC12, and MDCK cells were resuspended in 0.32M Sucrose, 10mM HEPES, 1mM MgCl₂, plus protease inhibitors, and passed through a ball bearing cell cracker. Post-nuclear supernatants (PNS) were obtained by recovering the supernatant of a centrifugation of 5min at 1000g. The recombinant light chains of TeNT and BoNT were produced and purified as in Galli

et al. (17). The PNS were treated with 200nM of the toxin indicated for 30min at 37°C in the resuspension buffer. The brevins were revealed by western blotting of the treated and untreated extracts with the anti-brevin antibody C110.1.

Immunocytochemistry

Cells were fixed with 3% paraformaldehyde (PFA) and processed for immunofluorescence as described (29). Confocal laser scanning microscopy was performed using a SP2 confocal microscope (Leica, Heidelberg, FRG). Images were assembled without modification using Adobe Photoshop (Adobe Systems, San Jose, CA).

Wound healing experiments

Cells were plated and cultured for 3 days to allow for the formation of monolayers. Cells were wounded by scratching with a bevel-edged needle of 0,6x 25mm (Terumo, Leuven, Belgium).

Time-lapse Video-Microscopy and velocity measurement

All studies were performed with an inverted microscope (Leica, Westlar, Germany) placed within a temperature controlled enclosure set at 37°C either with a 20x objective, 50ms exposure, at a rate of 20 exposures per hour, or with a 63x oil objective for stream acquisition of vesicle movement. In this case, DMEM without phenol red and without riboflavin (Invitrogen, Carlsbad, CA, USA) was used. We used a Cascade amplified camera (Roper Scientific) which allows an amplification of the transmitted signal up to 3000x. For vesicle observations, the amplification used was 2500x. The digital images were recorded and viewed using MetaMorph software (Universal Imaging Corporation, Downingtown, PA). Analysis of video sequences was done with MetaMorph and Excel (Microsoft, Redmond, WA).

After cell monolayer injuries, velocities were measured by the displacement of individual cells over time. A minimum of 60 cells were analyzed per condition (20 cells per injury: 10 at each border of the wound).

Statistical analyses were performed using the Kruskal-Wallis non-parametric test with statview software (SAS Institute Inc, NC).

Cell Adhesion Assays

All substrates were prepared by incubating overnight at RT, washing in PBS and saturating with PBS-BSA 1% (ultrapure BSA, Sigma): 2 µg collagen (rat tail collagen in 30% ethanol, Roche Diagnostics), 2 µg fibronectin (human fibronectin in borate buffer 0.1 M pH 8.1, PAA Lab., Linz, Austria), 2 µg mouse laminin (prepared from Engelbreth-Holm-Swarm mouse tumors, kindly provided by M. Vigny, in borate buffer 0.1 M pH 8.1) and 0.3 µg poly-ornithine (Sigma, in PBS) as a control. Alternatively, 1 µg anti-human Fcγ fragment-antibody (Jackson ImmunoResearch, West Grove, PA) was incubated overnight before coating with 0.5 µg E-cadherin-Fc chimera (human E-cadherin extracellular domain fused to human Fc fragment, Ecad-Fc, R&D Systems, Minneapolis, MN).

MDCK cells expressing the transgenes indicated were mechanically dissociated in PBS, BSA 1%, EDTA 0.5 mM and 5×10^5 cells were plated in 200 µl DMEM in 48-well tissue culture plates in the presence or absence of 10% FCS (PAA Lab.) Cells were allowed to adhere to the plates at 37°C for the times indicated, then the medium was entirely removed, the cells were washed three times with 1 ml PBS and dissociated with 100 µl trypsin for direct cell counting. Three independent experiments were carried out; in each case, two different cell clones were used in duplicate for each transgene. For immunofluorescence experiments, the cells were plated on collagen-coated glass coverslips and cultured for the indicated times at 37°C, washed with PBS, fixed and processed for immunofluorescence as above.

Antibody uptake experiments

Monolayers of MDCK cells were wounded by scratching. 2 h later cells were incubated for 1.5 h in DMEM 10% FCS containing 5 µg/ml of anti β1-integrin antibody. Cells were then fixed and processed for immunofluorescence as previously described. In three independent experiments, cells from both sides of wounds were scored for intracellular labeling (cells comparable to the cells marked with a * in figure 5C were considered positive for intracellular labeling). A minimum of 450 cells per wound were counted, total cells (at least 1750 cells per condition).

Results

Tetanus neurotoxin impairs cell migration

We first investigated whether the v-SNARE cellubrevin was expressed in MDCK cells. Indeed this is the case as a 12kDa band was recognized by the monoclonal antibody C110.1, previously shown to specifically detect cellubrevin and the neuronal synaptic v-SNAREs synaptobrevin 1/2 (14) (Figure 1A). Furthermore, canine Cb (cCb), as well as human Cb (hCb) and rat synaptobrevin 2 (Syb), is sensitive to TeNT, because treatment of MDCK cell extract with TeNT cleaved the 12kDa band recognized by C110.1.

In order to study the dynamic localization and function of Cb in MDCK cells, we used a stable cell line expressing GFP-Cb (27). We found that GFP-Cb containing vesicles were largely present throughout the lamellipod of MDCK cells treated with HGF (Figure 1B) thus suggesting that Cb may play a role in cell migration or at least in some function of the lamellipods of migrating cells. In order to study the function of Cb-mediated trafficking in cell migration we expressed the light chain of wild-type (wt) or inactive (E234Q, mut) TeNT (14) in our stable GFP-Cb expressing MDCK cell line (27). 24 hours treatment of the transfected cells with HGF revealed no obvious change in phenotype, as both the cells expressing wt-TeNT and mut-TeNT showed large lamellipods (figure 1C). Cells expressing the mutated form maintain their punctate labeling whereas cells expressing wt-TeNT have a diffuse GFP staining, owing to the cleavage of Cb and the resulting release of the GFP into the cytoplasm. This enabled us to directly detect the cells expressing wt-TeNT in videomicroscopy experiments (Figure 1D and movie 1). We then tested the ability of TeNT-transfected epithelial cells to heal a mechanical wound. As a first approach, we studied the migration of GFP-Cb expressing cells after transient transfection with wt-TeNT. We found a striking difference between cells showing a punctate pattern for GFP-Cb, thus being TeNT-negative, and those showing a soluble pattern, thus being TeNT-positive, as the latter did not seem to participate in the closing of the wound (figure 1D, online supplementary movie 1). As we were concerned that this could be the result of overexpression, we generated double stable cell lines co-expressing GFP-Cb and either wt-TeNT or mut-TeNT. Stable cell lines were selected on the basis of the GFP pattern - punctate when GFP-Cb was uncleaved, soluble when it was cleaved - and staining for TeNT using a monoclonal antibody raised against the light chain of TeNT (figure 1C). We repeated the wound healing experiments in the resulting double stable cell lines using two independent clones expressing wt- TeNT and two independent clones

expressing mut-TeNT (Figure 2A and B, movie 2). In cell tracking experiments, we were able to show that the expression of wt-TeNT resulted in a decrease in the migration speed of approximately 50% ($p < 0.0001$ for each pair of clones expressing wild-type versus inactive TeNT, the same p is obtained when the two clones of each category are pooled). Indeed, GFP-Cb/wt-TeNT expressing cell lines migrated at a speed of approximately $8\mu\text{m/h}$ whereas GFP-Cb/mut-TeNT cell lines migrated at a speed of approximately $17\mu\text{m/h}$ (Figure 2C). These results demonstrate that Cb plays an essential role in MDCK cell migration.

GFP-Cb dynamics in migrating cells

The study of GFP-Cb dynamics in migrating cells showed several types of behaviour: 1- immobile vesicular structures, 2- highly mobile vesicles and 3- transient accumulations at the leading edge. As an example of case 2, two mobile vesicles which are reaching the plasma membrane are indicated by an arrow or circled (Supplementary figure 1A and B, and online supplementary movie 3). Interestingly, we found that some of these transient accumulations of GFP-Cb at the leading edge did not move with the leading edge but became immobile while the lamellipod moved forward (figure 3A and supplementary movie 4). Quantification of the fluorescence intensity showed that there was a moderate increase of fluorescence in these GFP-Cb domains, followed by an abrupt decline in fluorescence as the leading edge advanced (our unpublished observations). Such transient accumulations were not seen in GFP-expressing cells and did not have the same dynamics as DiI, a lipidic marker used to follow the dynamics of the entire plasma membrane (our unpublished observations), thus they are not likely to result from the dynamics of membrane ruffles. Instead, these spots were reminiscent of focal contacts and in fact we found colocalization of some accumulations of GFP-Cb with Talin and Focal Adhesion Kinase (FAK) by confocal microscopy of immunostained fixed MDCK cells. However, in these domains GFP-Cb seems to concentrate at the distal extremity of the focal contact (figure 3B). These results indicated that Cb may participate in cell migration by regulating the trafficking at focal contacts.

Tetanus neurotoxin impairs cell-substrate adhesion

The enrichment of GFP-Cb close to focal contacts prompted us to test the hypothesis that Cb may be implicated in cell adhesion by assaying the ability of the different clones to adhere to

different substrates. Cells interact with the extracellular matrix via integrins, a family of cell surface heterodimeric receptors composed of various α and β subunits (30). GFP-Cb/wt-TeNT expressing cell lines adhered faster to collagen-coated plates than GFP-Cb/mut-TeNT cell lines (figure 4A left) with a maximum difference after 2 hours in the presence of serum suggesting that the effect seen on migration may be related to modification in integrin expression or recycling. Interestingly, in the absence of serum, a known activator of integrin recycling (31), we found no more difference between cells expressing wt- and mut-TeNT thus suggesting that the effect seen in the adhesion to collagen may be linked to integrin recycling (figure 4A right). The adhesion to collagen is mediated by a variety of integrin dimers containing the $\beta 1$ subunits (32, 33). It has been reported that receptors implicated in adhesion to collagen also promoted adhesion to laminin while cells adhere to fibronectin with $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrin heterodimers (34, 35). Accordingly, we found a similar difference of adhesion on laminin- and fibronectin-coated plates at 2 hours after plating but no difference on poly-ornithine, to which adhesion is considered to be mediated by electrostatic interaction of membrane lipid (figure 4C). Interestingly, adhesion to an E-cadherin-Fc substrate was also accelerated in GFP-Cb/wt-TeNT expressing cell lines (figure 4B). This could be attributed to homophilic E-cadherin-Fc/ MDCK endogenous E-cadherin interaction or to E-cadherin-Fc/ MDCK $\alpha 2\beta 1$ heterophilic interaction. Indeed the collagen ligand $\alpha 2\beta 1$ has also been shown to interact with the cell-cell adhesion molecule E-cadherin (36).

We then asked if the effects observed on cell adhesion would affect the morphology of cells 2hrs after plating on collagen, the time point for which the difference was maximal in the adhesion assay (figure 4A). We found that cells expressing wt-TeNT are still round whereas cells expressing mut-TeNT show a normal spreading in these conditions. Such a strong difference was not seen 4 hours after plating (figure 4D). In order to demonstrate that the effect of TeNT was specifically due to the cleavage of Cb, we studied the spreading on collagen of wild type MDCK cells co-transfected by wt TeNT plus either GFPCb wt or GFPCb VW, a TeNT-resistant mutant of Cb (28). Cells co-expressing TeNT and GFPCb VW spread more on collagen than those co-expressing TeNT and GFPCb wt (figure 4E).

Altogether, these results suggest that Cb may regulate cell migration by mediating the fast recycling of integrins particularly $\beta 1$ integrin with an additional possible direct or indirect implication in E-cadherin dependent adhesion. We tested this hypothesis by comparing the endocytosis of an antibody directed against $\beta 1$ integrin in our cell lines. We first checked that

there is no significant difference in the expression of $\beta 1$ integrin between our cell lines (figure 5A). We found that GFP-Cb and endocytosed $\beta 1$ integrin (detected by antibody uptake) partially colocalized in cells located at the border of wounds (figure 5B). In similar antibody uptake experiments, the labeling at the plasma membrane was similar when we compared the different clones (figure 5C) but cells expressing wt- TeNT showed a much weaker intracellular signal than mut-TeNT expressing cells located at the border of a wound. The ratio of cells with punctuate intracellular staining of anti- $\beta 1$ integrin internalization (symbol * in figure 5C) was strongly reduced in cells expressing wt-TeNT (48.2% +/- 5%) compared to cells expressing mut-TeNT (72.4% +/- 7.64; three independent experiments, chi square P-value<0.0001). Altogether, these results suggest that TeNT inhibits the recycling of $\beta 1$ integrins.

Discussion

In this paper, we were able to demonstrate the involvement of the endosomal vesicular SNARE Cb in cell migration and integrin-dependent adhesion. This is supported by the following evidence: 1) Cb traffics in lamellipods of migrating cells, 2) impairment of Cb function by TeNT leads to a two fold reduction in the migration speed, and 3) TeNT treatment alters cell-matrix adhesion and spreading, and $\beta 1$ integrin recycling. Therefore, our findings strongly suggest that exo-endocytosis is a key process in cell migration and adhesion and that maximal migration speed requires a fine regulation of cell adhesion.

As highlighted in the introduction, several important functions have been attributed to Cb, particularly in the recycling of plasma membrane receptors (17) and in phagocytosis in macrophages (20, 21), a process in which endosomal membranes are targeted in a polarized fashion to the site of particle binding and engulfment. Here we identify another function of Cb and early endosomes in cell migration and adhesion. We show that Cb-dependent trafficking regulates cell-substrate adhesion to a specific set of substrates including collagen, laminin and fibronectin, all known to involve $\beta 1$ integrins (37). The expression of TeNT resulted in a decrease in the migration speed, a faster adhesion to collagen-, laminin-, and fibronectin-coated plates and a decrease in the recycling of $\beta 1$ integrin. Thus, Cb may regulate cell migration by promoting the fast recycling of proteins implicated in cell adhesion particularly $\beta 1$ integrins, thereby decreasing cell-substrate adhesion and enabling the lamellipod to move forward. Our data are reminiscent of findings on the cell adhesion molecule L1. Indeed, the neuronal form of L1 has an additional exon that encodes an AP-2 binding site thereby promoting fast recycling of the protein (38) and associated with a weaker adhesion than the non-neuronal form (39). Thus, our data are compatible with the concept that optimal migration is obtained with submaximal cell-matrix adhesion. The involvement of Cb in cell migration is in agreement with the need for the recycling of membrane proteins and receptors, as previously suggested (11, 13).

Previous work has showed that $\alpha v\beta 3$ integrin recycles via a rab-4 dependent mechanism and that expression of dominant-negative rab4 compromises $\alpha v\beta 3$ -dependent cell adhesion (35). Furthermore, Rab11 stimulates the recycling of $\beta 1$ integrins in HeLa cells (31). In agreement with these studies, we demonstrate here a role for the early endosomal v-SNARE Cb that partially colocalizes with rab 4 (40) and rab11 (41), in the recycling of $\beta 1$ integrins, and in cell adhesion and migration. Similar to the effect of the synaptobrevin 2 knock out which impairs

both exocytosis and endocytosis of synaptic vesicles (42), the elimination of Cb by TeNT may affect exocytosis or endocytosis of $\beta 1$ integrins or both. The present study suggests that Cb is required for the efficient fast recycling of plasma membrane proteins such as $\beta 1$ integrin in migrating cells. The fact that $\beta 1$ integrins are still expressed at the plasma membrane of TeNT-expressing MDCK cells indicates that $\beta 1$ integrins are also able to reach the plasma membrane in a TeNT-insensitive manner. Further work will be required to elucidate the coordination and regulation of these different pathways of $\beta 1$ integrin trafficking. Our results are reminiscent of previous work showing that FAK(-/-) fibroblast-like cells cultured from E8 embryos have a reduced motility, a rounder morphology, and a poorer spreading than FAK(+/+) cells, but nevertheless have an enhanced attachment to collagen and laminin (43). Thus our data suggest that Cb-dependent recycling of $\beta 1$ integrins may regulate the dynamics of focal adhesion contacts.

Our data also suggest that TeNT may have an effect on neuronal cell adhesion in addition to the well known block of neurotransmitter release. Several botulinum neurotoxins were shown to stimulate axonal sprouting (16). This phenomenon may result from the incapacity of the affected neurites to properly regulate their substrate adhesion. Furthermore, the recycling of synaptic vesicles was recently shown to involve talin (44), a protein known to be involved in the regulation of integrins (45), thus suggesting that our observations in MDCK cells may also have implications in neuronal cells.

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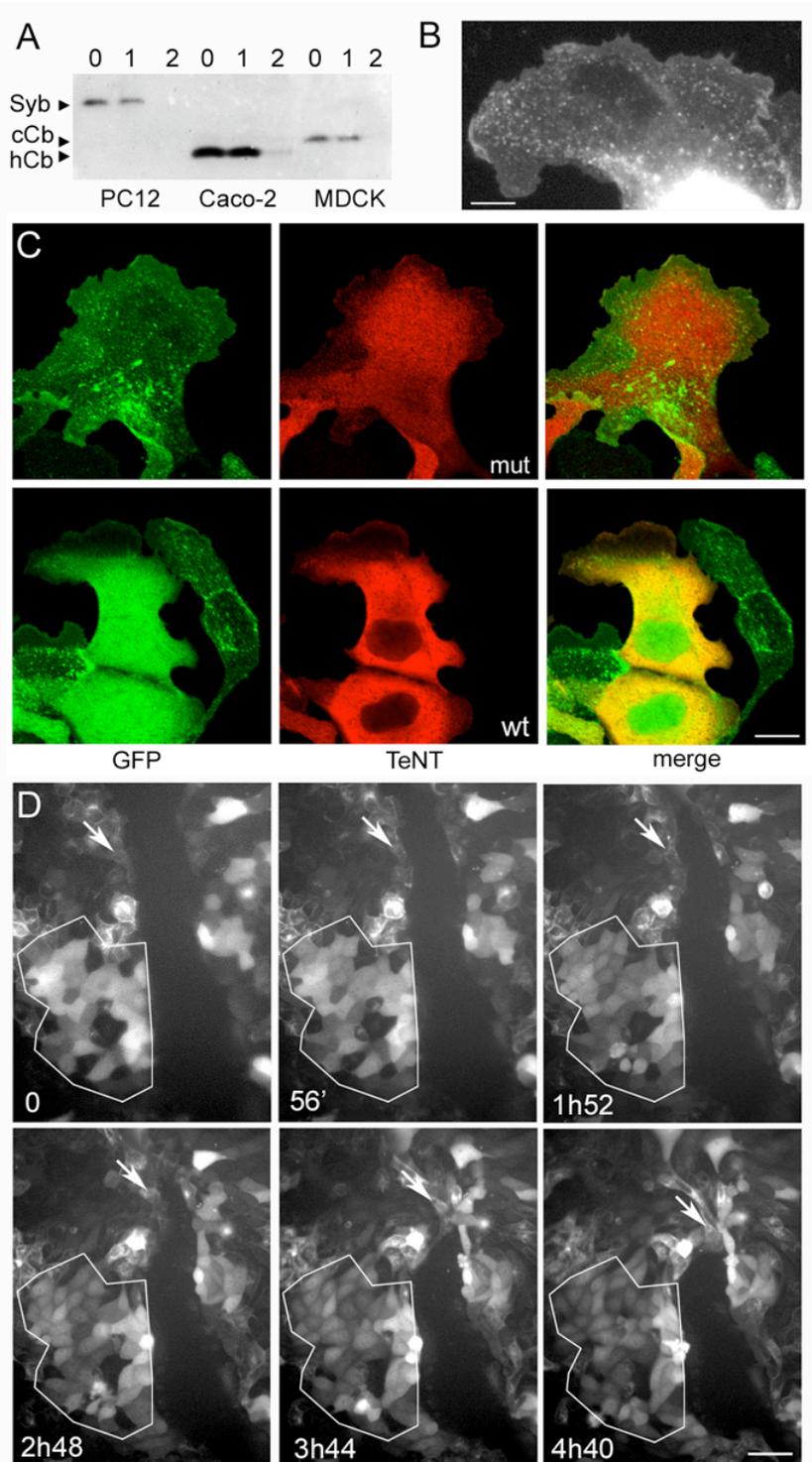


Figure 1

Tetanus neurotoxin cleaves canine cellubrevin and inhibits cell migration.

A- Western blot showing Synaptobrevin (Syb), human (hCb), and Canine (cCb) Cb in extracts from PC12 (rat cells), Caco-2 (human cells), and MDCK cells (canine cells) treated with Botulinum C1 (1) or Tetanus (2) neurotoxin in comparison to untreated cell extracts (0). HCb, cCb and Syb are TeNT-sensitive. Only one band (corresponding to cCb) is detected by C110.1, a pan-brevin monoclonal antibody, in MDCK cells. None of the brevins are sensitive to BoNT C1, as expected.

B- MDCK cells expressing GFP-Cb. GFP-Cb is localised in vesicles dispersed throughout the lamellipod Bar: 5µm.

C- MDCK expressing GFP-Cb and either mutant (mut) or wild type (wt) tetanus neurotoxin (red)

are both able to form lamellipods. Cells expressing mutant tetanus neurotoxin (TeNT) (top) contain vesicular GFP-Cb (green) whereas cells expressing wt-TeNT (bottom) show a diffuse labeling due to the cleavage of Cb and the liberation of soluble GFP. Bar: 13 μ m.

D- Cell border migration of MDCK cells expressing GFP-Cb, with or without coexpression of wt-TeNT, after monolayer injuries. Cells expressing wt-TeNT show a diffuse labeling of GFP due to the release of GFP by toxin cleavage. One area particularly rich in cells expressing wt- TeNT is outlined (bottom left sector). One cell not expressing wt-TeNT is indicated by an arrow. Note that this cell is moving faster than wt-TeNT expressing cells. These images were extracted from video 1 (online supplemental material), times indicated correspond to the time elapsed from the beginning of the film. Bar: 31 μ m.

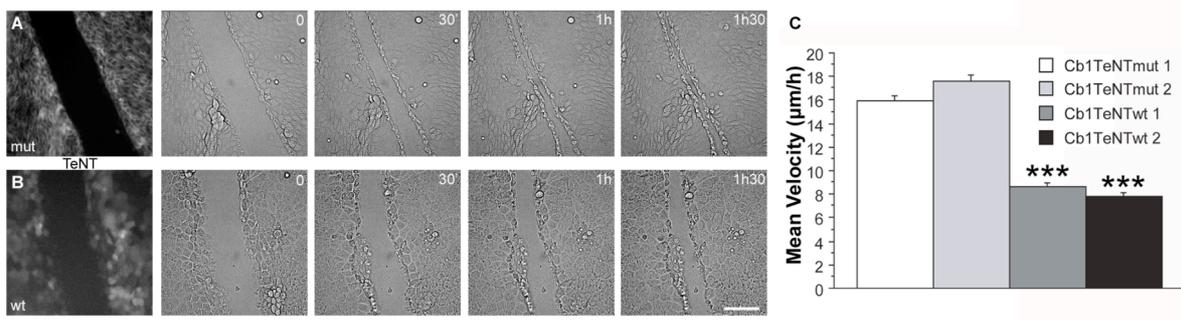


Figure 2

Stable expression of tetanus neurotoxin impairs cell migration

A- Cell border migration of MDCK cells co-expressing GFP-Cb and mut-TeNT after monolayer injuries.

B- Cell border migration of MDCK cells co-expressing GFP-Cb and wt-TeNT after monolayer injuries.

A, B These images were extracted from video 2 (online supplemental material), times indicated correspond to the time elapsed from the beginning of the film. The first image corresponds to the direct fluorescence pattern of the cells. Bar: 40µm.

C- The migration of MDCK cells expressing wt-TeNT is severely impaired. Quantification of the migration of MDCK cells expressing GFP-Cb and either wt- or mut-TeNT after monolayer injuries. (Cells counted: 60-80 per clone). Bars indicate standard deviation (***) p<0.0001).

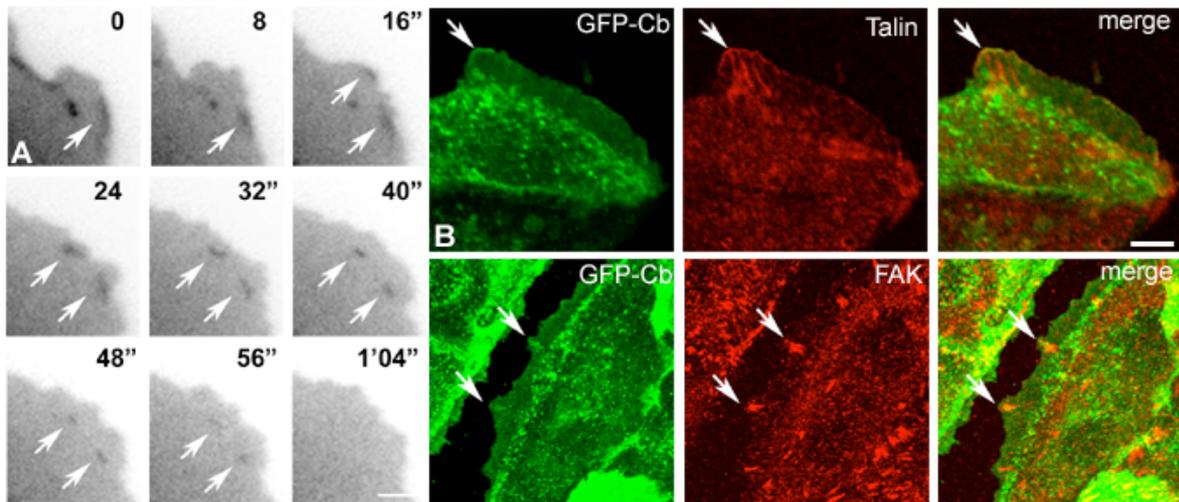


Figure 3

GFP-Cb labeled subdomains of lamellipod in migrating cells

A- A lamellipod of a migrating cell expressing GFP-Cb after a monolayer injury. Two transient accumulations of GFP-Cb are marked by arrows (time 0'', and 16''). These accumulations remain immobile while the leading edge moves forward (from 16'' to 56'') and then finally disappear (1'04''). These images were extracted from video 4 (online supplemental material), times indicated correspond to the time elapsed from the beginning of the film (' indicates min; '' indicates s). Bar: 2.7 μ m.

B- Colocalisation of GFP-Cb subdomains with the focal adhesion markers Talin (top) and Focal Adhesion Kinase (bottom). A concentration of GFP-Cb (green) colocalising with Talin (red) is indicated by an arrow (top panels). Two domains of colocalisation of GFP-Cb (green) with FAK (red) are also indicated by arrows (bottom panels). Note that in these domains, the GFP-Cb seems to be more distal than FAK. Bar: Top: 7 μ m, Bottom: 8.2 μ m.

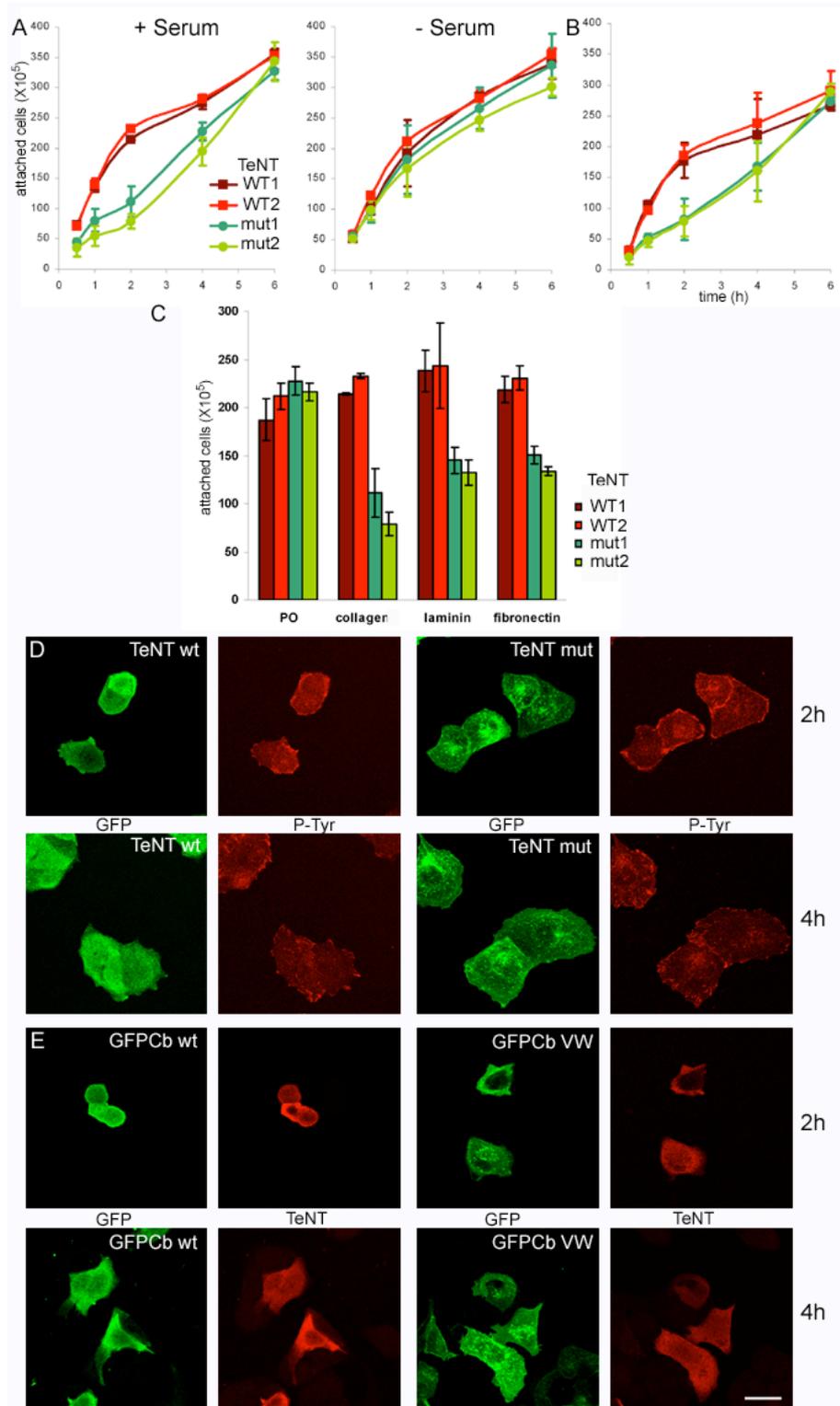


Figure 4
Cb regulates $\beta 1$ -integrin-dependent cell-substrate adhesion

A- Cells expressing wt-TeNT (red) adhere faster to collagen-coated plates than cells expressing mut-TeNT (green) in the presence of 10% serum (left panel) but not in absence of serum (right panel).

B- Cells expressing wt-TeNT (red) adhere faster to human Ecad-Fc-coated plates than cells expressing mut-TeNT (green).

A-B: Graphs represent numbers of cells adhered after different elapsed times. Values shown are mean \pm S.D. calculated from three independent experiments using the same cell clones; each experiment

was performed in duplicate.

C- Cells expressing wt-TeNT (red) adhere faster to collagen-, laminin- and fibronectin coated plates than cells expressing mut-TeNT (green). No difference was observed with poly-ornithinecoated plates (PO). Graph bars show mean values +/- S.D. calculated from three independent experiments at time 2h, the point with the highest difference between clones according to A and B; each experiment was performed in duplicate.

D- Cells expressing wt-TeNT spread slower on collagen than cells expressing mut-TeNT. Cells expressing wt-TeNT (upper panels, red, left) are more rounded and less spread and show a more diffuse Phospho-Tyr labeling (right) than cells expressing mut-TeNT (top panels, red) 2hrs after plating on collagen. No significant difference is seen 4hrs after plating (lower panel). In this experiment, anti-GFP labeling is detected in green and anti-Phospho-Tyr in red.

E- Expression of a TeNT resistant mutant of Cb (GFPCb VW) rescues the phenotype induced by TeNT (red). Cells expressing wt-TeNT and GFP-Cb VW (right) are more spread than cells coexpressing wt-TeNT and GFPCb (GFPCb wt, left), 2 hours after plating on collagen. DE, bar: 24 μ m

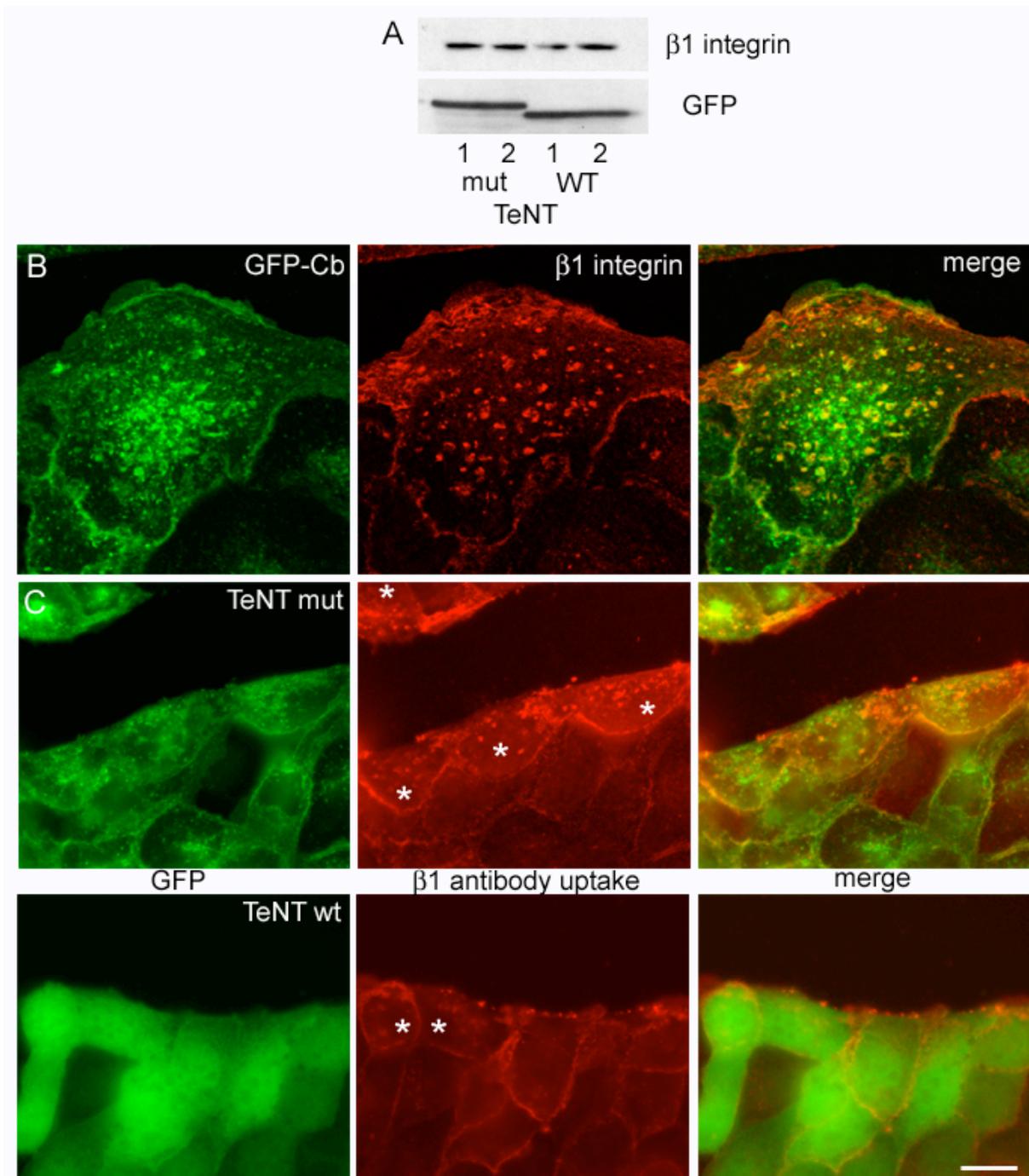


Figure 5

Cb regulates $\beta 1$ -integrin internalisation

A-There is no significant difference in $\beta 1$ integrin expression (upper panel) in cells expressing wt- or mut-TeNT. Western Blot showing the cleavage of GFP-Cb in cells expressing wt-TeNT (lower panel). B- $\beta 1$ integrin and GFP-Cb colocalise in cells co-expressing GFP-Cb (green) and mut-TeNT at the border of a wound after anti- $\beta 1$ integrin antibody uptake experiments ($\beta 1$ integrin labeling in red). (Bar: $39\mu\text{m}$).

C- Cells expressing wt-TeNT at the border of a wound (lower panel) show a less intense intracellular labeling after anti- $\beta 1$ integrin antibody uptake ($\beta 1$ integrin labeling in red) than cells expressing mut-TeNT. Cells with intracellular labeling as scored in our quantification are indicated with a *. (Bar: $20\mu\text{m}$).