Endocytosis is essential for a variety of cellular functions, including the internalization of nutrients and communication among cells, or between cells and their environment. Internalized molecules must be precisely sorted to their final cellular destinations to fulfill their specific function. Distinct endocytic pathways have been described to date, including clathrin-dependent endocytosis and caveola-mediated uptake, the mechanism giving access to the axonal retrograde pathway remains unknown. To investigate this sorting process, we examined the internalization of a tetanus neurotoxin fragment (TeNT Hc), which shares axonal carriers with neurotrophins and their receptors.

Previous studies have shown that the TeNT Hc receptor, which comprises polysialogangliosides, resides in lipid microdomains. We demonstrate that TeNT Hc internalization also relies on a specialized clathrin-mediated pathway, which is independent of synaptic vesicle recycling. Moreover, unlike transferrin uptake, this AP-2-dependent process is independent of epsin1. These findings identify a pathway for TeNT, beginning with the binding to a lipid raft component (GD1b) and followed by dissociation from GD1b as the toxin internalizes via a clathrin-mediated mechanism using a specific subset of adaptor proteins.
The TeNT receptor complex has been shown to comprise lipids and proteins (Montecucco et al., 2004). The polysialogangliosides GD1b and GT1b (Habermann and Dreyer, 1986; Lalli et al., 2003a), as well as one or more glycosylphosphatidylinositol (GPI)-anchored proteins (Herreros et al., 2001; Munro et al., 2001) are required for toxin binding to the neuronal surface. TeNT is associated with detergent-resistant membranes (DRMs), which are enriched in cholesterol and GPI-anchored proteins (Herreros et al., 2001), and its uptake is sensitive to cholesterol depletion (Herreros et al., 2001). Furthermore, pretreatment of neurons with phosphatidylinositol-specific phospholipase C to cleave GPI-anchored proteins from their lipid anchor prevents TeNT intoxication (Munro et al., 2001). Altogether, these findings suggest that TeNT follows a polysialoganglioside- and DRM-dependent route for its internalization in neuronal cells. However, in previous EM studies on spinal cord neurons, gold-labeled TeNT was detected in surface pits resembling clathrin-coated invaginations, as well as in coated and uncoated vesicles (Parton et al., 1987; Lalli et al., 2003b). Because clathrin-mediated internalization and the endocytosis of proteins associated with DRMs have been largely viewed as mutually exclusive (Parton and Richards, 2003), the association of TeNT with clathrin coats was unexpected.

To resolve this apparent paradox, we have studied the internalization machinery responsible for the uptake of TeNT into MNs using a C-terminal binding fragment of TeNT (Lalli et al., 2003a). In this study, we show that TeNT HC endocytosis in MNs is independent of SV recycling, the major route of internalization at the presynaptic terminal, and demonstrate that although TeNT HC binds to DRMs on the MN surface, it uses a clathrin-mediated pathway for its entry. This specialized clathrin- and AP-2–dependent uptake mechanism does not require the endocytic adaptor protein epsin1, further indicating that specific adaptors play important functions in initial sorting events during endocytosis.

**Results**

TeNT HC internalization in MNs is independent of presynaptic activity

Although previous studies implied that TeNT does not enter the NMJ via SV endocytosis (Habermann and Dreyer, 1986), some studies suggested that the toxin can take this route in brain-derived neurons, such as hippocampal neurons (Matteoli et al., 1996) and that it may enter SV-like vesicles in spinal cord neurons in culture (Parton et al., 1987). In light of these findings, we assessed whether SV exo/endocytosis is the physiological route of TeNT entry in MNs. Several lines of evidence indicate that this is not the case. First, we tested the colocalization of internalized TeNT HC and the SV protein VAMP-2. MNs were incubated with Alexa Fluor 555–TeNT HC at 37°C, fixed, and stained for VAMP-2. Under resting conditions, colocalization in the cell body, neurites, or synaptic contacts was very limited (Fig. 1, A and B). Moreover, stimulation of SV exo/endocytosis by depolarization did not increase the extent of colocalization (Fig. 1, A and B).

Figure 1. **TeNT HC internalization is independent of SV exocytosis and recycling.** (A) MNs were incubated with 20 nM Alexa Fluor 555–TeNT HC for 30 min at 37°C, either under resting conditions (a–d) or after stimulation of SV exo/endocytosis by adding 60 mM KCl to the medium just before TeNT HC addition (e–h), fixed, and stained for VAMP-2. Only very limited colocalization of TeNT HC and VAMP-2 under resting or stimulated conditions, which were quantified in B, was found. Error bars represent the SEM. (C and D) MNs were incubated with 15 nM BoNT/A and 2 nM BoNT/D for 22 h at 37°C to cleave SNAP-25 and VAMP-2. Untreated cells were processed in parallel for comparison. (C) Cells were scraped and analyzed by Western blotting using antibodies raised against the cleaved fragments of SNAP-25 and VAMP-2, as well as actin, as a loading control. (D) 20 nM b-TeNT Hc was added to MNs for 30 min at 37°C. MNs were shifted to ice, treated with MESNA before fixation, and stained for VAMP-2 (a and e), SNAP-25 (b and f), and biotin (c and g). Pretreatment with BoNTs did not affect TeNT HC internalization. DIC, differential interference contrast. Bars: (A) 5 μm; (D) 10 μm.

To further investigate the endocytic pathway of TeNT, we used a biotinylated, thiol-cleavable form of TeNT Hc (b-TeNT HC). By exposing intact neurons to cell-impermeable reducing reagents, such as 2-mercaptoethanol sulfonic acid (MESNA; Schmid and Smythe, 1991), biotin can be cleaved off surface-bound TeNT HC, while the internalized b-TeNT HC is protected. Staining for the remaining biotin allows the internalized TeNT HC to be detected selectively over the surface-bound TeNT HC even in thin structures such as axons. Biotinylation does not change the binding and internalization properties of TeNT HC because preincubation with a 100-fold excess of unlabeled toxin completely abolished the binding of b-TeNT HC to MNs (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200508170/DC1). Furthermore, under internalization...
conditions, b-TeNT H₃ colocalized with Alexa Fluor 555–TeNT H₃ (Fig. S1 B). Importantly, biotin could be cleaved off the surface-bound b-TeNT H₃ by treatment with MESNA on ice. In contrast, in cells incubated at 37°C the label remained cell-associated, showing that b-TeNT H₃ had been taken up in the soma and neurites (Fig. S1 C).

For an independent assessment of a role for SV recycling in TeNT H₃ uptake, we then preincubated MNs with botulinum neurotoxin (BoNT)/A and D for 22 h to block SV exocytosis, and, thus, recycling (Humeau et al., 2000). The samples were then incubated with b-TeNT H₃ for 30 min at 37°C, treated with MESNA on ice, fixed, and stained for VAMP-2 and SNAP-25, as well as for biotin, to visualize internalized TeNT H₃. The complete cleavage of SNAP-25 and VAMP-2 by BoNT/A and D was confirmed by Western blotting (Fig. 1 C) and by indirect immunofluorescence (Fig. 1 D), indicating that SV exo/endocytosis was blocked under these conditions. However, TeNT H₃ internalization was not affected in intoxicated MNs (Fig. 1 D, g) compared with untreated cells (Fig. 1 D, c).

TeNT H₃ uptake is dynamin-dependent

We next used the b-TeNT H₃ to test the requirement for dynamin in this process. Dynamin is a GTPase essential for clathrin- and caveolin-mediated endocytosis, as well as for several other endocytic and vesicle-trafficking events. Incubation of MNs with the cell-permeable peptide P4, which inhibits dynamin function (Marks and McMahon, 1998), but not with the scrambled
peptide P4S, significantly reduced uptake of TeNT HC, whereas its overall binding to the neuronal surface was not affected (Fig. 2 A). These results were confirmed by overexpressing the well-characterized dynamin mutant K44A, which is defective in GTP binding and hydrolysis and restrains invaginated pits from pinching off (Fig. 2 B; Danke et al., 1994). We used microinjection to introduce foreign DNA into MNs because lipid-based transfection reagents abolished axonal transport in MNs. In contrast, microinjection of plasmids driving the overexpression of control proteins had no effect on cell viability and retrograde transport (Deinhardt and Schiavo, 2005). Expression of dynamin2K44A significantly reduced TeNT HC endocytosis at the level of both the soma and neurites (Fig. 2 B), without affecting its binding to the MN surface (Fig. 2 C). These results indicate that dynamin is a central player in TeNT HC internalization and rule out differences in the mechanism of uptake of TeNT HC between axons and the soma. This is important because, topologically, only the axon is physiologically relevant for TeNT HC uptake and retrograde transport. A total block of TeNT HC endocytosis by the expression of dynamin2K44A was seen in >95% of the cells (Fig. 2 D). However, dextran internalization still took place under these conditions (Fig. 2 A, b) or upon P4 treatment (Fig. 2 A, b), confirming that MNs were viable and still capable of endocytosis via dynamin-independent pathways. We chose dextran as a control marker for internalization because we found that cholera toxin subunit B (CTB), which is another widely used marker for clathrin-independent endocytosis, is internalized in a strictly dynamin-dependent fashion in MNs (unpublished data). This was surprising because CTB has been shown to use a dynamin-independent entry pathway in other cell types, such as HeLa and mouse embryonic fibroblasts (Torgersen et al., 2001; Massol et al., 2004; Kirkham et al., 2005; Glebov et al., 2006).  

**TeNT HC localizes to clathrin-coated pits**

In previous studies, TeNT was found in coated pits, as well as in coated and uncoated vesicles in spinal cord neurons (Parton et al., 1987; Lalli et al., 2003b). Furthermore, TeNT is taken up by a clathrin-independent route in nonneuronal cells (Montesano et al., 1982). Therefore, we decided to investigate whether TeNT internalization is strictly clathrin-mediated in cultured MNs. First, we assessed the colocalization of clathrin and TeNT HC at the light microscopy level in MNs microinjected with GFP–clathrin light chain (CLC). Upon incubation with TeNT HC at 37°C, we could see only a partial overlap between GFP-CLC and TeNT HC, more readily in the cell body than in the axon (Fig. 3, A and B).

To verify this colocalization at a fine structural level, we used TeNT HC coupled with HRP. Just like b-TeNT HC, binding of this fusion protein to MNs was inhibited by preincubation with an excess of unlabeled TeNT HC and, upon internalization, Alexa Fluor 488–TeNT HC and HRP–TeNT HC showed extensive colocalization (Fig. S2, A and B, available at http://www.jcb.org/cgi/content/full/jcb.200508170/DC1). The relationship between internalized TeNT HC and clathrin-coated structures was then investigated at early stages of the internalization process. To this end, we synchronized the uptake of HRP–TeNT HC by preincubating the MNs at 4°C and, subsequently, warming to 12°C. This low-temperature treatment allows the plasma membrane to invaginate but inhibits vesicle–pinching off (unpublished data). The effectiveness of this protocol was...
confirmed by incubating MNs with either b-TeNT Hc or HRP–TeNT Hc at 12°C and, subsequently, treating cells with MESNA or performing the DAB reaction in the presence of ascorbic acid, which is a membrane-impermeable inhibitor of the HRP staining (Stoorvogel, 1998). Upon MESNA treatment, no biotin was detectable by immunofluorescence (Fig. S2 C). Similarly, when the DAB reaction was performed in the presence of ascorbic acid on cells incubated at 12°C, we observed immunolabeling of clathrin, but no DAB staining (Fig. S2 D). These findings indicated that the coated pits remained open to the external medium at 12°C and confirmed the suitability of this temperature block for the study of the initial stages of endocytosis.

After 12°C incubations, the electron-dense DAB reaction product generated by HRP–TeNT Hc was readily observed in coated pits on the plasma membrane of soma, dendrites, and axons (Fig. 3 C). The nature of these coated domains was confirmed by immunogold staining with clathrin heavy chain (CHC) antibodies, which labeled pits containing HRP–TeNT Hc (Fig. 4 A). The DAB reaction product found in both shallow pits and in deeper invaginations was closely associated with clathrin lattice components (Fig. 3 C, a–b’). After the 12°C block, we allowed MNs to internalize TeNT Hc at 18°C to monitor its intracellular axonal transport. Fine structural analysis suggests that progression along the endocytic pathway was slowed down at this temperature and lead to an increase of early endosomal carriers. At 18°C, HRP–TeNT Hc was found in coated vesicles (Fig. 3 C, c) and other vesicular and tubular structures within the axon.

Gold immunolabeling of coated pits in axons was not easily discerned in thin EM sections, as permeabilization of these structures and access to the antigen appeared to be impaired because of the highly packed cytoskeleton in these areas. Therefore, we decided to prepare whole-cell mounts by extracting neurons with Triton X-100 before fixation, thereby improving the antigen availability and providing a better overview over the total population of TeNT Hc–positive membranes. To stabilize HRP–TeNT Hc–containing pits and protect them from solubilization, DAB cross-linking was performed before detergent extraction. At 12°C, the vast majority of DAB-positive structures located along the axons (Fig. 4 B, a, b, and d) and on the cell body (Fig. 4 B, c) were clathrin positive.

The ganglioside GD1b does not enter coated pits in complex with TeNT Hc
Polysialogangliosides of the b-series, including GD1b and its analogues GT1b and GQ1b, have previously been described as...
essential components of the TeNT receptor complex (Kitamura et al., 1999). However, these lipids, like other residents of sphingolipid-rich microdomains, are thought not to enter clathrin-coated pits (CCPs; Nichols, 2003). Therefore, we asked where GD1b localizes on the neuronal surface in relation to TeNT Hc and clathrin-coated invaginations. By light microscopy, we were able to confirm colocalization of TeNT Hc and GD1b on the neuronal surface by using a specific anti-GD1b antibody (MOG-1; Fig. 5 A). Furthermore, preincubation of MNs with MOG-1 inhibited the binding of TeNT Hc in a dose-dependent manner (Fig. S3 A, available at http://www.jcb.org/cgi/content/full/jcb.200508170/DC1), confirming that GD1b is an essential component of the TeNT receptor complex. To obtain a higher resolution view of the association between TeNT Hc and polysialogangliosides, we incubated MNs with HRP–TeNT Hc in the presence of noncompeting concentrations of MOG-1 at 12°C (Fig. S3 B) and analyzed the samples by EM. As previously described for other components of lipid microdomains, we found clusters of gold-labeled GD1b on the cell surface, often in close proximity to the DAB precipitate generated by HRP–TeNT Hc (Fig. 5 B). In addition, the DAB precipitate was frequently associated with coated structures (Fig. 5 B, arrows and arrowheads). However, we were unable to detect GD1b in CCPs containing the DAB cross-linking product. Instead, gold-labeled GD1b

Figure 5. GD1b and TeNT Hc undergo independent sorting on the plasma membrane. MNs were probed with TeNT Hc and an anti-GD1b antibody and analyzed at light and EM levels. (A) Cells were incubated with the antibody MOG-1 (2 μg/ml) and 20 nM Alexa Fluor 555–TeNT Hc for 30 min on ice, followed by 5 min at 22°C, and then were washed, fixed, and imaged. A good overlap of the two signals could be seen both on neurites (a–d) and soma (e–h). DIC, differential interference contrast. (B) EM analysis. MNs were incubated on ice with 60 nM HRP–TeNT Hc and 10 μg/ml MOG-1, followed by 10 nm immunogold. After washing, samples were shifted to 12°C for 45 min (a–h) or 37°C for 15 min (o). MNs were fixed and incubated with DAB/H2O2 to label HRP–TeNT Hc. The anti-GD1b gold-labeled antibody is excluded from endocytosed HRP–TeNT Hc vesicles (asterisks), clathrin lattices (arrows), and invaginated coated pits (arrowheads). Bars, 0.2 μm.
was frequently found at the edge of HRP–TeNT H₃C–positive pits (Fig. 5 B, a–h). Furthermore, internalized gold particles were very rarely detected upon incubation at 37°C, suggesting that GD1b remains surface-bound (Fig. 5 B, i). Under the same conditions, TeNT H₃C was identifiable in many vesicles and tubules, all of which were free of GD1b gold label (Fig. 5 B, i).

To confirm that the DAB precipitate did not conceal any gold particles in internalized structures, we incubated MNs with HRP–TeNT H₃C together with gold-conjugated TeNT H₃C. Under these conditions, gold label was clearly visible in all HRP-positive structures (Fig. S2 E).

A subset of clathrin endocytic adaptors is required for TeNT H₃C endocytosis

We next examined whether CCP formation is required for TeNT H₃C internalization by affecting specific components of the clathrin-dependent endocytic machinery. The blockade of transferrin uptake, which strictly relies on a clathrin-mediated pathway (Harding et al., 1983), was taken as a positive control, whereas the CTB entry, which occurs via clathrin-independent routes in several cell types (Torgersen et al., 2001; Nichols, 2003; Kirkham et al., 2005), allowed us to monitor the specificity of the inhibition. In control MNs, both CTB and b-TeNT H₃C readily entered neurons in soma and neurites (Fig. 6, a and c).

Expression of the phosphorylation-deficient mutant of the AP-2 subunit μ₂ (μ₂T156A), which is incorporated into AP-2 complexes but cannot be phosphorylated at Thr156, thus, impairing AP-2–dependent clathrin-mediated endocytosis (Olusanya et al., 2001), blocked the uptake of TeNT H₃C (Fig. 6 d), as well as transferrin internalization (not depicted). In contrast, CTB entry in μ₂T156A–expressing MNs is barely altered (Fig. 6 f), suggesting that, in contrast to that observed in hippocampal neurons (Shogomori and Futerman, 2001), its mechanism of uptake in MNs is mainly clathrin independent. As expected, binding of TeNT H₃C to the cell surface was not affected by expression of μ₂T156A (not depicted). Similar results were obtained by expressing a truncation mutant of the accessory protein AP180 (AP180-C). This mutant inhibits uptake of EGF and transferrin in COS-7 cells (Ford et al., 2001). Expression of AP180-C inhibited both TeNT H₃C (Fig. 6 g) and transferrin endocytosis (not depicted), whereas CTB internalization was not visibly affected (Fig. 6 i).

In contrast to AP-2 and AP180 dominant-negative constructs, expression of a mutant version of the adaptor protein epsin1 had no significant effect on TeNT H₃C internalization (Fig. 7 A, d and g). This epsin1R63L,H73L mutant is unable to bind phosphatidylinositol-4,5-bisphosphate (PtdIns[4,5]P₂) and blocks transferrin uptake in COS-7 cells (Ford et al., 2002). As expected, transferrin endocytosis was completely inhibited in epsin1R63L,H73L-expressing MNs (Fig. 7 A, f). These findings were confirmed by an independent EM analysis, where expression of this epsin1 mutant abolished the uptake of transferrin-HRP (not depicted), leaving the internalization of HRP–TeNT H₃C unaffected (Fig. 7 B, arrows). In addition, epsin1R63L,H73L-expressing cells showed no obvious morphological alterations and displayed occasional CCPs and clathrin-coated vesicles (CCVs; Fig. 7 B, arrowheads and insets). These findings, together with previous works reporting the existence of AP-2-independent
routes (Nesterov et al., 1999; Conner and Schmid, 2003; Hinrichsen et al., 2003; Motley et al., 2003; Lakadamyali et al., 2006), suggest that different subsets of adaptors proteins functionally define distinct clathrin-dependent pathways.

To investigate the spatial relationships of TeNT Hc, transferrin, and epsin1 during the early stages of endocytosis, we analyzed the distribution of epsin1 by immuno-EM in MNs incubated with gold-conjugated TeNT Hc and transferrin-HRP at 12 or 20°C. Under these conditions, we could detect TeNT Hc in clathrin-coated structures either containing or devoid of transferrin, as well as transferrin-containing pits and vesicles devoid of TeNT Hc (Fig. S4 A, available at http://www.jcb.org/cgi/content/full/jcb.200508170/DC1). Quantitative analysis of all TeNT Hc-containing structures revealed that 54% of these were devoid of transferrin. Interestingly, less than half of these TeNT Hc single-positive structures labeled for epsin1, whereas two-thirds of the TeNT Hc and transferrin dual-positive vesicles and pits were also positive for epsin1 (Fig. S4 B). Therefore, epsin1 accumulates preferentially, but not exclusively, on transferrin-containing structures. It should be considered, however, that permeabilization with digitonin is likely to affect the stability of HRP-negative membranes (TeNT Hc ± epsin1). Therefore, these structures are likely to be underestimated, as observed by independent experiments in which the occurrence of transferrin-HRP and HRP–TeNT Hc containing CCP and CCV has been quantified (unpublished data).

The effects of the disruption of different components of the clathrin-dependent machinery on TeNT Hc uptake are evident in the quantitative analysis provided in Fig. 7 C. Although TeNT Hc endocytosis into MNs can be blocked by disruption of the
clathrin adaptors AP-2 and AP180, it does not require the adapt-
ator protein epsin1. Given that epsin1 is targeting ubiquitinated
receptors to the late endosomal/lysosomal pathway (Le Roy and
Wrana, 2005), the independence of TeNT Hc internalization
from epsin1 function is in agreement with the finding that TeNT
Hc escapes targeting to acidic compartments and degradation in
MNs (Lalli et al., 2003a; Bohnert and Schiavo, 2005).

Discussion

An open question in the field of membrane trafficking is how
distinct extracellular ligands following internalization via the
same endocytic pathway (i.e., CCPs, caveolae), are sorted in
eyear endosomes to their different intracellular destinations.
In neurons, this process is crucial for the targeting of growth
factors and their receptor complexes to short- and long-range
trafficking routes, ultimately leading to diverse and often op-
posite biological functions. This is exemplified by the action
of nerve growth factor, which has been shown to alter growth
cone dynamics by local signaling, while it acts as a survival
factor following axonal retrograde transport and transcriptional
activation in the nucleus (Miller and Kaplan, 2001). The fine
balance between these two processes is fundamental for our
understanding of differentiation, synaptogenesis, and plasticity in
the nervous system.

TeNT Hc was chosen as a tool to monitor endocytosis
in MNs based on its high binding affinity to neuronal mem-
branes (Lalli et al., 2003a) and its ability to enter the same axo-
nal transport compartment used by nerve growth factor (Lalli
and Schiavo, 2002) and the neurotrophin receptor p75NTR
(unpublished data). Previous work highlighted the association
of TeNT with both coated and uncoated structures, whereas at
later time points it was found in many endocytic organelles, in-
cluding coated and uncoated vesicles, tubules, and SV-like profiles
(Montesano et al., 1982; Parton et al., 1987; Matteoli et al.,
1996; Miana-Mena et al., 2002; Lalli et al., 2003b; Roux et al.,
2005). However, a functional analysis assessing the role of the
various internalization routes has yet to be made.

We show that clathrin-dependent internalization plays
a nonredundant role in the uptake of TeNT in MNs. At 37°C,
CCPs and CCVs that are positive for TeNT are rarely found,
especially along axons. To explore if the localization to coated
structures is representative for the entire TeNT Hc pool, we low-
ered the temperature to inhibit fission, thus, trapping forming
pits on the cell surface. A striking colabeling of TeNT Hc and
clathrin was seen under these conditions. Importantly, whole-
mount EM analysis revealed that the HRP–TeNT Hc–containing
areas along the axon were positive for clathrin, indicating that
TeNT Hc does enter MNs via CCPs. In this light, the uncoated
structures containing TeNT Hc observed previously (Schwab
and Thoenen, 1978; Parton et al., 1987; Lalli et al., 2003b) may
represent vesicles from which the clathrin lattice was rapidly
removed (Blanpied et al., 2002; Ehrlich et al., 2004).

Several virulence factors, such as cholera and Shiga tox-
in, are taken up by clathrin-dependent and -independent routes
(Sandvig and van Deurs, 2002; Parton and Richards, 2003;
Saint-Pol et al., 2004), which may display different extents of
cross talk and redundancy in various cell types (Torgersen et al.,
2001). To ensure that clathrin-dependent endocytosis is the
main, nonredundant route of TeNT Hc internalization and iden-
tify the endocytic machinery responsible for its uptake, we used
dominant-negative constructs interfering with distinct steps of
coat recruitment and/or pinching off from the plasma membrane.
Impairing dynamin function led to a block in TeNT Hc
uptake, showing that this GTPase is necessary for TeNT Hc
endocytosis. In addition, disruption of the clathrin machinery
by mutants of AP180 nearly completely abolished TeNT Hc
endocytosis. Furthermore, the uptake of TeNT Hc, as well as
of transferrin in MNs, are strictly AP-2–dependent, confirming
previous findings obtained with transferrin in other cell types
(Hinrichsen et al., 2003; Motley et al., 2003; Lakadamyali et al.,
2006). Thus, a functional clathrin machinery is strictly required
for TeNT Hc endocytosis (Fig. 8).

In contrast, expression of a dominant-negative epsin1 mu-
tant did not interfere with TeNT Hc internalization. Epsin1 is
an endocytic adaptor for clathrin-dependent and -independent in-
ternalization that binds to ubiquitinated receptors via its ubiqui-
tin interacting motifs (Chen and De Camilli, 2005; Le Roy and
Wrana, 2005; Sigismund et al., 2005). It has a modular structure
comprising binding sites for PtdIns(4,5)P2, CHC, AP-2, and
other accessory factors for clathrin-mediated endocytosis.
The mutant used in our study is deficient in PtdIns(4,5)P2 binding
(Ford et al., 2002) and was reported to block endocytosis by
sequestering AP-2. However, we observed opposite effects with
the AP-2 µ2 and the epsin1 mutants on TeNT Hc uptake in
MNs, suggesting that their inhibitory activity may not com-
pletely overlap. In contrast with that reported in COS-7 cells,
we did not detect aggregation of AP-2 in MNs or glial cells
expressing epsin1R63L H73L (Fig. S5, available at http://www.jcb.
org/cgi/content/full/jcb.200508170/DC1). In these conditions,
uptake of transferrin into MNs was blocked by expression of
this epsin1 mutant, indicating that epsin1R63L H73L displays a
dominant-negative effect in these cells that is likely to be in-
dependent of AP-2 sequestration.

Epsin1 function overlaps with that of the homologues
Eps15 or Eps15R, as shown by the limited effect of single and
double knockdown on transferrin and EGF internalization in
HeLa cells (Huang et al., 2004). In contrast, transferrin uptake
was completely blocked by expression of epsin1R63L H73L, dem-
onstrating that epsin1 has a nonredundant function in a subset
of clathrin-mediated, AP-2–dependent endocytic events in MNs
(Fig. 8). Moreover, the block of transferrin internalization by
epsin1R63L H73L suggests that epsin1 acts at an early step of the
uptake process, before pit closure, implying that sorting in the
endocytic pathway initiates at the plasma membrane. In this re-
gard, we found epsin1 to be associated preferentially, but not
exclusively, with transferrin-containing CCPs and CCVs.
In agreement with this view, the sorting of endocytic cargos
internalized via clathrin-mediated uptake, such as low- density
lipoprotein and influenza virus, to distinct population of
endosomes has been shown to begin at the level of CCPs
(Lakadamyali et al., 2006).

In spite of its entry into MNs via a clathrin-dependent
mechanism, TeNT Hc binds to DRMs, and its uptake can be
Internalization pathways in MNs. Cholera toxin uptake is mediated by a classical clathrin-dependent internalization route occurring in soma and dendrites. SV exo/endocytosis accounts for the majority of endocytic events at the presynaptic terminal and may involve multiple clathrin-dependent steps. In contrast, CTB, which binds to GM1 clustered in lipid rafts, is internalized by a clathrin-independent, dynamin-dependent mechanism in MNs. TeNT HC exploits a pathway requiring lipid rafts and the clathrin machinery, which is distinct from aforementioned routes of internalization. At the NMJ, TeNT HC binds to a lipid-protein receptor complex containing the ganglioside GD1b. TeNT HC is then laterally sorted into CCPs and, during this sorting event, GD1b is excluded from the toxin receptor complex. Internalization of TeNT HC is dependent on dynamin, AP-2, and AP180, but does not require epsin1. Once internalized, TeNT HC is targeted to a stationary early sorting compartment (Lakadamyali et al., 2006), to which other endocytic routes may converge. This early sorting compartment is functionally coupled to the axonal retrograde transport pathway.

Materials and methods

Reagents

Chemicals were obtained from Sigma-Aldrich, BDH Chemicals Ltd., or Invitrogen, unless otherwise stated. Sulfo-NHS-SS-biotin and EZ-link–activated maleimide-HRP were purchased from Pierce Chemical Co. Antibodies 9E10, X22, and 12CA5 were obtained from the Cancer Research UK antibody facility, antibody against the C-terminus of SNAP-25 was a gift from O. Rossetto (University of Padova, Padova, Italy). The epsin1 antibody was a gift from L. Traub (University of Pittsburgh, Pittsburgh, PA). The IgG3 mouse monoclonal antibody MOG1 reacts with 8 M), and GD2, but not with GT1b, GG1b, or GD3 (Boffey et al., 2005). Plasmids encoding dynamin1RNA, epsin1T156A, and AP180 T176A were a gift from H. McMahon (Laboratory of Molecular Biology, Cambridge, UK). AP-2 μ2T186A was a gift from E. Smythe, and GFP-CLC was a gift from L. Greene and Bohnert and Schiavo, 2005). These findings indicate that clathrin adaptors are assembled in a cargo-selective manner to drive the internalization of plasma membrane proteins and their ligands (Owen et al., 2004; Lakadamyali et al., 2006). This process has, in turn, the power to generate different populations of early endosomes, which have different targeting determinants and fates within the cell.
Protein labeling
To couple TeNT-Hc to HRP, 10 nmol of cysteine-tagged TeNT-Hc were incubated with 5 mM EDTA and 6.5 mg EZ-link-activated maleimide-HRP in PBS overnight at 4°C. The conjugate was purified first on a ConA-Sepharose (GE Healthcare) and eluted with 0.2 M α-methylmannoside in 10 mM sodium phosphate, pH 7.2. HRP–TeNT-Hc was bound to NINTA-agarose (QIAGEN) and eluted in 20 mM Heps-NaOH, pH 8.3, 150 mM NaCl, and 500 mM imidazole. Samples containing HRP–TeNT-Hc were pooled and dialyzed against PBS. To double label TeNT-Hc with an Alexa Fluor dye and HRP, fluorescent labeling was performed first, according to the manufacturer's instructions, using half of the recommended amount of dye and without the addition of glutathione to stop the reaction. Alexa Fluor–labeled TeNT-Hc was dialyzed against PBS to remove the excess dye before HRP-conjugation.

Microinjection and internalization assay
MN cultures were prepared and maintained in culture as previously described (Bohnert and Schiavo, 2005). Cells were injected with 0.05 mg/ml of plasmid between 4 and 7 d in vitro. In cases of microinjection of multiple plasmids (e.g., the pTRE2 T156A plasmid that requires a transactivator pTf for expression; CLONTECH Laboratories, Inc.), 0.04 mg/ml of each construct were mixed before injection. MNs were incubated with 15–20 nM TeNT-Hc and then either biotinylated or Alexa Fluor–labeled for 30 min at 37°C. In selected experiments, 20 μg/ml Alexa Fluor 594–transferin, 10 ng/ml Alexa Fluor 555–CTB, or 0.2 mg/ml tetramethylrhodamine dextran (mol wt 3,000) were mixed with TeNT-Hc before addition to the cells. 60 μM KCl was added to the medium just before addition of the ligands to test the effects of depolarization.

In experiments where MNs were pretreated with P4 or P4-scrambled peptide (Marks and McMahon, 1998), 50 μM of peptide was added to the medium at 37°C for 2 h before incubation with 20 nM Alexa Fluor 488–TeNT-Hc and 0.2 mg/ml tetramethylrhodamine dextran.

For MESNA treatment, MNs were cooled on ice and then incubated three times for 15 min with 15 mM of ice-cold MESNA in neurobasal medium (Invitrogen), pH 8.3. Cells were washed three times in neurobasal medium and once in PBS, and then fixed. To test the effect of SV exo/endoctyosis on TeNT-Hc uptake, MNs were seeded on 13-mm coverslips. At 6 d in vitro, MNs in two wells were incubated with 15 mM BovNT/A and 2 mM BovNT/D for 2 h at 37°C, while control wells were left untreated. Coverslips from treated and untreated wells were then threaded into a new dish and incubated with 20 nM TeNT-Hc for a further 30 min at 37°C before treatment with MESNA on ice, fixing, and processing as described in the following paragraph. The remaining cells from each well were washed in PBS, scraped, centrifuged, and then resuspended in SDS sample buffer. Proteins were analyzed by Western blotting using standard procedures. Antibodies were used as follows: anti–VAMP-2 (69.1), 1:500; anti–SNAP-25, 1:300; anti–SNAP-25, 1:1,000; and anti–VAMP-2 [69.1], 1:500; anti–SNAP-25, 1:1,000; anti–actin (AC-40), 1:1,000; and anti-HA [12CA5], 1:1,000; anti-Myc [9E10], 1:250; secondary anti–mouse (Jackson), 1:500; secondary anti–rabbit (Jackson), 1:500; secondary anti–goat (Jackson), 1:1,000; and secondary anti–donkey (Jackson), 1:1,000. Proteins were analyzed by Western blotting from each well were washed in PBS, scraped, centrifuged, and then resuspended in SDS sample buffer. Proteins were analyzed by Western blotting using standard procedures. Antibodies were used as follows: anti–VAMP-2 (69.1), 1:500; anti–SNAP-25, 1:300; anti–SNAP-25, 1:1,000; and anti–VAMP-2 [69.1], 1:500; anti–SNAP-25, 1:1,000; and anti–actin (AC-40), 1:1,000; and anti–HA [12CA5], 1:1,000; anti–Myc [9E10], 1:250; secondary anti–mouse (Jackson), 1:500; secondary anti–rabbit (Jackson), 1:500; secondary anti–goat (Jackson), 1:1,000; and secondary anti–donkey (Jackson), 1:1,000. Proteins were analyzed by Western blotting from each well.

For whole mounts, MNs were treated with DAB (0.75 mg/ml in 50 mM Tris-HCl, pH 7.4) and with ascorbic acid buffer (20 mM Heps-NaOH, 70 mM NaCl, and 50 mM ascorbic acid, pH 7.0) at 5°C for 30 min after treatment with HRP–TeNT-Hc and chased in medium. Cells were then permeabilized with 40 ng/ml digitonin in permeabilization buffer (25 mM Heps-KOH, 38 mM aspartate, 38 mM glutamate, 38 mM gluconate, 2.5 mM MgCl2, and 2 mM EGTA, pH 7.2), fixed in 2% PFA, quenched with 50 mM glycine, and blocked with 1% BSA before treatment with primary antibody in PBS containing 1% BSA for 60 min at room temperature. To enhance the signal, intermediate species-specific antibodies were used. MNs were washed and incubated with an appropriate 10-nm gold-labeled secondary antibody (British Biocell International) in 2% BSA and 2% FCS in PBS for 45 min at room temperature. After washing, cells were fixed and processed for conventional EM. In double- and triple-label experiments, MNs were incubated with 80 nM HRP–TeNT-Hc or with 20 μg/ml transferrin-HRP together with TeNT-Hc directly conjugated to 10-nm gold particles (as described by Ondrizzi et al., 1996) in serum-free neurobasal medium for 45 min at 4°C. Cells were washed and shifted to 12 or 20°C before incubation with DAB/H2O2.

Online supplemental material
Fig. S1 shows biotinylated TeNT-Hc as a probe to study membrane trafficking in MNs. Fig. S2 shows characterization of HRP–TeNT-Hc. Fig. S3 shows that binding of TeNT-Hc to MNs can be competed by preincubation with a specific anti-GD1b antibody. Fig. S4 shows distribution of TeNT-Hc, epitope, and transferrin in the endocytic pathway of MNs. Fig. S5 shows that overexpression of epispin1163,1072 does not lead to AP-2 aggregation in spinal cord cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200508170/DC1.

We are thankful to S. Tooze, A. Behrens, and members of the Molecular Neuropathobiology laboratory for critical reading of the manuscript.

This work was supported by Cancer Research UK (K. Deinhardt and G. Schiavo), Wellcome Trust 063034 (H.J. Willison), and the Medical Research Council (O. Berninghausen and C.R. Hopkins).

Submitted: 25 August 2005
Accepted: 6 July 2006

References

References


