Endosome fusion induced by diphtheria toxin translocation domain

Antonella Antignani and Richard J. Youle*

Biochemistry Section, Surgical Neurology Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, 35 Convent Drive MSC 3704, Bethesda, MD 20892

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Endosomal cargo travels through a dynamic vesicle network en route to degradation by lysosomes or recycling through the Golgi apparatus back to the cell surface. Rab5 is a key determinant of the early endosomes by organizing effector proteins in specific subdomains and mediating early endosome fusion. We find that early endosome morphogenesis and maturation is disrupted by diphtheria toxin (DT). Rab5 bound endosomes increase in size and in Rab5 content due to luminal toxin exposure, whereas Rab7 positive endosomes are not detectably altered. These changes appear to be caused by an activity of the toxin entry domain (T domain) as mutations inactivating either the receptor binding (CRM107) or ADP-ribosyl transferase (CRM197) activities do not inhibit the effect of DT on endosome morphogenesis. In contrast, mutations in the T domain or diminishing the endosomal pH gradient, which prevents T domain membrane insertion, inhibit these endosome changes. The change in size appears to be due to changing the early endosome fission-fusion equilibrium. The Rab5 membrane exchange rate, assessed with photoactivatable GFP-Rab5, decreases in the presence of DT. These changes to endosomes may reflect activities of the T domain that mediate toxin entry to the cytosol. The nontoxic mutant DT, CRM197, yields a new tool to manipulate endosome dynamics in living cells.

early endosomes | Rab5 | Rab7

Many toxic proteins of bacterial and plant origin enzymatically modify substrates within the cytosol of mammalian cells (1). These toxins translocate certain protein domains across the plasma membrane or, after endocytosis, across the membrane of intracellular organelles by a number of different mechanisms. Diphtheria toxin is synthesized and secreted by *Corynebacterium diphtheriae* as a single chain polypeptide containing three distinctly different functional domains: the amino-terminal enzymatic “A chain,” the translocation or “T domain,” (residues 202–378), and the carboxyl-terminal receptor binding domain (residues 386–535). Diphtheria toxin (DT) binds to a cell surface receptor and becomes endocytosed into clathrin-coated vesicles. After vesicle uncoating, DT is delivered to early endosomes and is proteolytically cleaved by furin to generate a disulphide-linked, heterodimeric A chain-B chain form of the toxin. The low pH in endosomes generated by a proton-pumping vacuolar ATPase (V-ATPase) induces all three toxin domains to form partially unfolded molten globule-like structures, thereby exposing hydrophobic helices in the T domain that insert into the endosomal membrane and mediate the translocation of the partially unfolded A chain across the endosomal membrane. Once in the cytosol, the free A chain refolds into its biologically active conformation and inhibits protein synthesis by the ADP-ribosylation of EF-2, leading to the death of the cell (2, 3).

How the A chain crosses the endosomal membrane into the cytosol remains unclear. The structure of native DT shows that the T domain consists of a bundle of 10 α -helices (4). Six of these helices fold similarly to helices of the Bcl-2 family members (5) and the channel-forming domains of certain colicins (6). The two long hydrophobic helices (TH8 and TH9) buried within the native T domain are thought to insert into the membrane bilayer

upon exposure to the low pH of endosomes (7–9), and several other helices adhere along the luminal side of the membrane (10). Studies in planar lipid bilayers have shown that under low pH conditions the T domain is capable of translocating the A chain across the bilayer in the absence of other proteins (11). However, Ratts *et al.* (12) provide evidence that the translocation of DT A chain out of purified early endosomes is not autonomous as it requires the addition of a cytosolic translocation factor complex composed in part by Hsp90 and thioredoxin reductase.

Studying the kinetics of toxin entry, Hudson and Neville (13) conclude that the rate-limiting step in protein synthesis inhibition by DT is not the ADP-ribosylation of EF-2 by cytosolic toxin but rather the entry of DT into the cytosol. They propose a model in which DT enters the cytosol in a bolus of sufficient size to rapidly inactivate all EF-2 in that cell. The size of a single bolus of DT calculated from data over the range of 10^{-11} to 10^{-9} M DT was found to remain constant at ≈ 20 molecules. They suggest that the cytosolic entry mechanism of DT results from a unique ability of the internalized toxin molecules to destabilize the vesicular membrane resulting in the simultaneous release of all of the toxin molecules in an endosome. The process by which a destabilized vesicle releases its toxin content to the cytosol compartment may be thought of as a functional lysis of the membrane vesicle barrier, but the actual physical process of toxin release from endosomes is unknown. Intracellular membrane traffic transfers materials within vesicular compartments to other membrane-bounded compartments through membrane destabilizing reactions that permit fusion of vesicles with the target cellular compartments.

Rab proteins, which constitute the largest family of monomeric GTPases and their effectors coordinate consecutive stages of membrane transport, such as vesicle formation, vesicle and organelle motility, and tethering of vesicles with target membranes during the endocytic and exocytic pathways. These proteins are highly compartmentalized, making them excellent markers of vesicle subtype (14). Rab5 is localized on the membrane of early endosomes, where it regulates clathrin-coated vesicle-mediated transport from the plasma membrane to the early endosomes and homotypic early endosome fusion through the recruitment of a large number of effector proteins (15, 16). As early endosomes become acidified, they form the first cellular environment suitable for DT translocation to the cytosol.

Here, we report the real-time visualization of endosomes during DT entry and the consequences of DT translocation on the status of the endosomal membranes. By analysis of the dynamics of endosomes, using Rab5 as a marker, we identify

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*To whom correspondence should be addressed. E-mail: youler@ninds.nih.gov.

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Fig. 1. Endosome changes in the presence of DT. (A–C) HeLa cells, transfected with a plasmid encoding EYFP-Rab5 for 12–15 h, were incubated in the absence (A) or presence (B) of 0.5 nM DT (A and B) or CRM197 (C) for 30 min at 37°C. The cells were examined by confocal microscopy and the calculation of the diameter of Rab5a positive endosomes was performed by using Volocity software. (Scale bars: 10 μ m.) (D) The histogram represents the distribution of the calculated diameters of Rab5 positive endosomes ($n = 2,000$) chosen randomly in control and toxin treated cells, and the black curves represent the Gaussian distribution of the histogram data.

dramatic consequences on endosome size due to the DT translocation domain insertion into membranes.

Results

Early Endosome Morphology in the Presence of DT. Subsequent to HeLa cell surface receptor binding, DT, at a concentration (1 nM) that causes a 90% reduction in protein synthesis after 4 h (17), enters transferrin (Tf) positive endosomal compartments within 5 min and is progressively concentrated over 30 min into vesicles with characteristics of the degradative pathway (18). To probe potential alterations in the endosomal compartment during the DT membrane translocation process, we visualized HeLa cells transiently expressing EYFP-Rab5 after exposure to DT. Substantial overexpression of Rab5 was shown to cause pronounced enlargement of the early endosomes (19, 20). In our experiments, EYFP-Rab5 was expressed at sufficiently low levels that the enlargement was not observed (Fig. 1A). Under these conditions, we found that cells incubated with 0.5 nM DT for 30 min display enlarged Rab5 positive vesicles (Fig. 1B) compared with untreated cells (Fig. 1A). A mutation of glycine 52 to glutamate in the DT catalytic domain (CRM197) causes the loss of the ADP ribosylation activity and cytotoxic activity (21). We found that CRM197 also induces the formation of enlarged Rab5 positive vesicles (Fig. 1C), showing that the effect on endosomes is not a consequence of DT inhibition of protein synthesis. When we analyzed the distribution of the diameters of Rab5 positive endosomes ($n = 2,000$) chosen randomly in control and toxin treated cells, we found that the major fraction of endosomes

from DT and CRM197 cells had diameters in the interval of 0.7–1.2 μ m, whereas, in control cells, most endosomes had a diameter between 0.4 and 0.6 μ m. We measured also a clear increase in the proportion of endosomes with a diameter $>1 \mu$ m in DT and CRM197 treated cells compared with untreated cells (Fig. 1D).

This DT-induced alteration in morphology of the vesicles does not require Rab5 overexpression because we detected enlarged endosomes in untransfected cells treated with DT and immunostained with anti-Rab5 antibody (data not shown). These enlarged Rab5 positive vesicles stain positive for another marker, EEA1, confirming that they represent early endosomes. The quantification of the colocalization of Rab5 with EEA1, represented as a Pearson's correlation coefficient, indicates less association between Rab5 and EEA1 in control cells compared with that in the early endosomes in DT and CRM 197 treated cells [supporting information (SI) Fig. S1]. This is likely due to the higher number of enlarged endosomes in DT treated cells compared with the control cells.

We compared the percentage of Rab5 fluorescent volumes distributed in three diameter ranges in control and toxin treated cells (Fig. S2). We considered the fluorescent volumes with a diameter $<0.5 \mu$ m as one group that would include the coated and uncoated vesicle populations (22), those vesicles with a diameter between 0.5 and 1 μ m and those vesicles with a diameter $>1 \mu$ m. We found that in cells treated with wild-type DT or CRM197, almost 60% of EYFP-Rab5 positive endosomes have a diameter $>1 \mu$ m compared with 30% in control cells (Fig. S2). The toxin ricin, that transits early endosomes after caveolae mediated endocytosis before translocation to the cytosol from within the ER, did not affect vesicle diameter (Fig. S2). We also analyzed the populations of Rab5 positive vesicles in cells incubated with a chemical conjugate of human transferrin (Tf) and a diphtheria toxin mutant (CRM107) with a 10,000-fold reduced affinity for the DT receptor (Tf-CRM107) (23). This immunotoxin binds and enters cells via the transferrin receptor before translocation across membranes via the DT T domain. Like DT and CRM197, this toxin increases the population of Rab5 positive vesicles with diameters $>1 \mu$ m in diameter, indicating that the effect does not depend on DT receptor binding (Fig. S2). Thus, DT mutants either in the receptor binding domain or in the enzymatic domain retain endosome enlargement activity.

Mutations E349K and P308S in the T domain inhibit DT membrane translocation (23–25). We individually inserted these two mutations in an inactive version of the DT with a point mutation into the catalytic domain (E148S) (26, 27). We expressed and purified DT-E148S (DTs), DTsE349K, and DTsP308S and examined their effect on the early endosomes. The percentage of Rab5 fluorescent volumes distributed in three diameter ranges in control and cells treated with each of the three recombinant toxins was compared with that of wild-type DT and CRM107. Recombinant DTs is able to induce endosome enlargement similar to wild-type DT and CRM197 whereas the two mutants, DTsE349K and DTsP308S, were largely deficient in this activity supporting the model that the DT T domain is responsible for endosome enlargement (Fig. 2A and B).

Low pH in endosomes is required for the translocation of DT by triggering a conformational change in the T domain that exposes hydrophobic domains and initiates toxin membrane insertion (28, 29). Lysosomotropic agents, such as NH_4^+Cl^- , that raise the endosomal pH by reducing the proton gradient across the membrane block DT toxicity (30). To test whether the endosomal enlargement induced by DT depends on the insertion of the T domain into the endosomal membrane we incubated HeLa cells with 1 mM NH_4^+Cl^- in the presence of DT or CRM197 and determined the size of the Rab5 positive endosomes. In the presence of NH_4^+Cl^- , $\approx 70\%$ of the endosomes in

cells treated with DT and CRM197 had a diameter $<1\ \mu\text{m}$, similar to that of control untreated cells (Fig. 3*A* and *B*). There is, however, a 10% increase in the population of vesicles with diameters $<1\ \mu\text{m}$ (Fig. 3*A* and *B*) due to NH_4^+Cl^- alone. This indicates that the increase in endosomal size occurs due to T domain insertion into early endosomes and not at later stages of the toxin trafficking pathway. When HeLa cells were treated with nocodazole to depolymerize microtubules and inhibit the transport of the endosomal carrier vesicles (ECV) to late endosomes (31), although the control endosomes were smaller, DT retained the capacity to enlarge them (data not shown).

We considered the possibility that the endosome enlargement induced by the T domain reflects an activity associated with DT translocation. If T domains act cooperatively to mediate translocation, a prediction consistent with the bolus model, additional T domain activity supplied from inactive DT molecules, such as that in CRM197, may increase DT toxicity. Measuring protein synthesis inhibition in Vero cells by different DT concentrations in the presence or in the absence of CRM197 revealed that CRM197 at a concentration below that which competes for receptor binding increased cell sensitivity to DT 50-fold (Fig. 3C). This indicates that T domains function cooperatively, consistent with the model that the change in the endosome morphology is correlated to the delivery of the DT catalytic domain into the cytosol.

A dominant negative Rab5 mutant, S34N, inhibits fusion

Fig. 3. Endosome perturbation alters toxin activity. (A and B) HeLa cells, transfected with the plasmid encoding EYFP-Rab5 for 12–15 h, were incubated without or with 0.5 nM diphtheria toxin or CRM197 for 30 min at 37°C (A) in the presence or absence of 5 mM NH₄Cl (A and B). (Scale bars: 10 μm.) The calculation of the diameters of Rab5 positive endosomes was performed considering 80% of all transfected cells per well for each conditions using Volocity software (B). (C) Vero cells (1 × 10⁶ cells) were incubated with different concentrations of DT in the absence (open squares) or in the presence (filled squares) of 1 × 10⁻¹² M CRM197. Cells were incubated with the toxins for 3 h, and the protein synthesis inhibition was measured as described in ref. 24.

Fig. 4. Role of Rab5 in endosome fusion. (A) HeLa cells, transfected with the plasmid encoding EYFP-Rab5S34N for 12–15 h, were incubated with 0.5 nM diphtheria toxin or CRM197 for 30 min at 37°C. (B) The calculation of the diameters of Rab5 positive endosomes was performed considering 80% of transfected cells for each conditions, using Volocity software. (Scale bars: 10 μ m.)

analysis of the diameters of $\approx 30,000$ fluorescent endosomes considering 80% of the total transfected cells per experiment (Fig. 4B). Thus, the effect of DT on endosome size requires Rab5 activity.

Dynamics of Rab5 Association with Single Endosomes in Living Cells.

Rab5 levels dynamically fluctuate on individual early endosomes during fusion and fission events that eventually, over time, link all endosomes into a network (32). The early endosomes, containing cargo destined for lysosomes, through repetitive fusion events, accumulate this cargo into fewer and larger endosomes, which are rapidly removed from the early-endosomal network via the loss of Rab5 and the concomitant acquisition of Rab7 (32). We asked whether DT can alter the dynamic network of this endocytotic pathway and whether there is a change in Rab5 dynamics along with toxin induced enlargement of Rab5 positive endosomes. We used Rab5 fused to a photoactivable form of GFP (PAGFP), a mutant of the *Aequorea victoria* GFP (33), that makes it possible to select proteins in specific regions of interest and analyze the kinetics of their localization within cells.

We tracked 50 photoactivated endosomes (1–2 endosomes per transfected cell) from cells incubated with and without DT and measured the fluorescence intensity of each endosome every 20 s. We confirmed that photoactivated endosomes in control cells expressing PAGFP-Rab5 were the same size as those in EGFP-Rab5 expressing cells, whereas photoactivated endosomes in DT treated cells were larger and displayed a higher level

of PAGFP-Rab5 fluorescence (Fig. 5A–C). We found that in control cells the amount of Rab5 associated with a given endosome fluctuated considerably over 5 min, as shown by a significant decrease in GFP-Rab5 fluorescence and by the termination of almost half of the analyzed traces before 5 min (Fig. 5C). As seen in the study in ref. 32, fission of Rab5 positive tubules below levels of detection and release of Rab5 from the membrane could explain the decrease.

PA-GFP-Rab5 displayed a higher level of fluorescence intensity on the large endosomes in DT treated cells and was more stable than the PAGFP-Rab5 on control endosomes (Fig. 5B and C). Comparing the average fluorescence intensity with time after photoactivation of Rab5 on endosomes (Fig. 5D) revealed that the fluorescence of Rab5 on the DT and CRM197 treated endosomes diminished more slowly over time than Rab5 on the control endosomes. The Rab5 fluorescence dropped off almost completely after 5 min in control cells, whereas endosomes from DT and CRM197 retained 40% of the initial fluorescence value. A high level of Rab5 loss causing track terminations resulted in the larger standard deviation values in the control endosomes. The track terminations occurred less frequently in DT treated cells. Because Rab5 positive endosomes are brighter and bigger, it was possible track them during the entire time series (5 min). Interestingly, from the analysis of the traces over 5 min, we found that, in contrast to the differential loss of Rab5 fluorescence, the variation in the endosome diameter is similar in DT and CRM197 containing vesicles relative to the control (Fig. S3). Thus, the greater endosome size in DT treated cells appears to reflect the greater retention of Rab5 on DT endosomes. This could be due to less fission of tubules or less Rab5 release that maintains greater fusion activity.

Rab7, another member of the Rab GTPase protein family, has been localized to late endosomes and plays a key role downstream of Rab5 in regulating membrane transport leading from early to late endosomes (34, 35). Therefore, we examined Rab7 to see whether it is present in these enlarged endosomes in toxin treated cells and found no difference in the diameter or morphology of Rab7 positive endosomes between control cells and cells treated with DT or CRM197 (data not shown). Thus, DT appears to affect early endosomes upstream of Rab7 by inhibiting the exchange of Rab5 with Rab7.

Discussion

A remaining gap in understanding DT activity is the process of translocation of the A chain across membranes to the cytosol. Early endosomes are the first sorting station from which endocytosed materials are directed to various cellular destinations and they represent the crucial compartment along the endocytic pathway from which the DT catalytic domain productively crosses into the cytosol. Using fluorescently labeled Rab5, a regulatory GTPase that mediates maturation of endosomes, we have shown that DT induces an enlargement in the diameter of the early endosomes and this enlargement is correlated to its cytotoxic activity. The enlargement of the endosomes depends on the DT T domain, because mutations in the translocation domain abolish this alteration in the endosomal morphology, whereas mutations either in the receptor binding domain or in the catalytic domain do not inhibit this activity of DT. Raising the pH in the endosomal compartment blocks the DT-induced enlargement of endosomes, indicating that acidity-driven conformational change of the T domain in the endosome lumen is required for this activity. Vesicle swelling may be induced by DT translocation due to a flux of low molecular weight molecules from the cytosol. However, because Rab5 inhibition eliminates the early endosome enlargement, we suggest that DT perturbs the morphology of endosomes by either increasing the rate of early endosome fusion or decreasing the rate of fission.

Fig. 5. Dynamics of the early endosomes in the presence of DT. (A and B) HeLa cells were transfected with PAGFP-Rab5 for 24–28 h and incubated in the absence (A) or presence (B) of 0.5 nM CRM197 for 30 min at 37°C. To photoactivate PAGFP, ROIs marked with yellow, red, and white arrows were irradiated with a 405-nm laser. PAGFP-Rab5 was imaged by using 488-nm laser excitation before (pre) and 30 s after (post) 405-nm light photoactivation. Images of two photoactivated endosomes (marked with the yellow and red arrows) were captured every 20 s for 5 min as series of z sections covering the entire thickness of the cell (A and B), showing the endosomes labeled with the red arrows (B Center), yellow arrows (B Right), and white arrows (data not shown). (C and D) Changes in the fluorescence intensities within photoactivated endosomes were measured over the time (C), and percentages of initial PAGFP-Rab5 fluorescence were plotted versus time (D). The data represent the mean $\pm$ SD of 50–55 endosomes during time-lapse measurements from control cells and cells incubated with DT or CRM197 (D). (Scale bars: 10 μ m.)

Endosomes receive incoming vesicles from the plasma membrane. Recycling cargo exits in Rab4-positive tubules, which frequently grow out and detach from Rab5-positive cores through constant nucleation of Rab4 domains. Cargo destined for late endosomes, such as DT, is retained in individual endosomes. Thus, by parallel sorting activities and interconnectivity of endosomes, degradative cargo gradually accumulates in fewer and bigger endosomes. Rab5 vesicles convert to Rab7 vesicles, removing the Rab7 vesicles from the early-endosomal network and rendering them fusion-competent with late endosomes, although homotypic fusion events can temporarily restore Rab5 levels. We have shown that DT is able to both increase the level of excessively fused endosomes and maintain endosomes in a Rab5-rich state consistent with the promotion of homotypic early endosome fusion. Using photoactivable GFP-Rab5, we found that DT slows down the rate of extraction of Rab5 from endosomal membranes. The increased fusion and the inhibition of the subsequent maturation of the early endosomes by DT may allow the accumulation of the toxin in the early endosome until it reaches the “quantum” (13) for release into the cytosol.

The Bcl-2 family of proteins, important regulators of programmed cell death in multicellular organisms (36), have two central hydrophobic α -helices surrounded by four amphipathic α -helices, an α -helix organization similar to that of the DT T domain (5). As with the structurally related helices 8 and 9 of DT that insert into membranes at low pH, helices 5 and 6 of Bcl-xL and Bax are hypothesized to insert into membrane bilayers during apoptosis (37). Interestingly, recent results show that Bax (38) and Bcl-xL (39) can mediate fusion of mitochondria. Thus, the membrane inserting T domain of DT may share an activity to activate membrane fusion processes with Bcl-2 family members albeit acting differentially on endosomes and mitochondria, respectively.

How endosome fusion may participate in the native toxicity of DT remains unclear. We have analyzed the protein synthesis inhibition activity of DT in HeLa cells stably expressing GFP-Rab5 and GFP-Rab5S34N and found that Rab5 transfected cells were

more susceptible to the action of the toxin than the Rab5S34N expressing cells (data not shown). However, for unknown reasons, both cell lines were more susceptible than control vector expressing lines. These initial results suggest that promotion of endosome fusion could be correlated with the cytotoxic effect of DT. Nevertheless, DT entry into cells increases Rab5 retention on endosomes and thereby causes their enlargement. This activity occurs after membrane insertion of the T domain and is correlated with membrane translocation process suggesting that DT crosses into the cytosol from these unusual endosomal structures.

Materials and Methods

Plasmids and Cell Culture. The EYFP-Rab5 plasmid was provided by H. McBride (University of Ottawa, Ottawa), and the EYFP-Rab5 S34N was provided by B. Ceresa (University of Oklahoma, Norman).

The Rab5 cDNA, digested with KpnI and BamHI (New England Biolabs), was cloned into PAGFP-C1 vector, digested with KpnI and EcoRI. HeLa cells were grown in DMEM and 10% FBS. They were transfected by using the FuGENE (Roche) and treated as indicated in individual experiments 12–15 h after transfection with the EYFP constructs or 24–28 h after transfection with PAGFP-Rab5. DT and CRM107 were purchased from List Biological Laboratories. Tf-CRM107 was synthesized as reported in ref. 23.

Confocal Microscopy and Image Analysis. Cells were grown in two-well chambers for confocal microscopy. For immunofluorescence, cells were fixed for 30 min in 4% paraformaldehyde in PBS and were stained with anti-EEA1 or anti-Rab5 antibody (BD Biosciences) followed by staining with goat anti-mouse Alexa Fluor 568 antibody (Molecular Probes). Images were captured with a model LSM 510 microscope (Carl Zeiss MicroImaging), using a 63 $\times$ or 100 $\times$ Apochromat objective (Carl Zeiss MicroImaging). Projected series of z sections collected with intervals of 0.5–0.75 μ m between sections are shown. For photoactivation of PAGFP-Rab5, ROIs were selected as the smallest circle shape allowed by the image acquisition program. The same intervals between optical section were using for imaging. Postacquisition processing and analysis were performed with Volocity image analysis software (Improvision). For the analysis of PAGFP-Rab5, endosomal ROIs were selected in the first image collected after photoactivation (postactivation values). The same regions were transferred without change to the image obtained before photoactivation.

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- PNAS | June 10, 2008 | vol. 105 | no. 23 | 8025