Effects of dynamin inactivation on pathways of anthrax toxin uptake

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Internalization and traffic to acidic endosomes of anthrax lethal factor (LF) and protective antigen (PA), bound to the anthrax toxin receptor (ATR), is required for LF translocation into the cytosol, where it can elicit its toxic effects. Dynamin is required for clathrin-mediated endocytosis, and long-term disruption of dynamin function blocks internalization of PA.

We have used LFn-DTA, a surrogate of LF consisting of the N-terminal domain of LF fused to the catalytic subunit of diphtheria toxin, to differentiate the effects of acute and long-term block of dynamin function on LFn-DTA toxicity. Both forms of interference reduce LFn-DTA toxicity only partially, consistent with alternative routes for LFn-DTA endocytosis. In contrast, a long-term block of dynamin activity results in a further interference with LFn-DTA toxicity that is consistent with an altered endosomal environment, probably an increase in endosomal pH.

Endocytosis, the uptake by a cell of soluble molecules, membrane-associated proteins, and membrane-bound ligands, may occur through several pathways. The most prominent entry route is mediated by clathrin, which forms coats that engulf vesicles at the plasma membrane. John Heuser's remarkable images of clathrin coat formation have profoundly affected our understanding of membrane vesiculation . Assembly of a coat, containing clathrin as well as a number of other proteins, is required for the early steps of membrane deformation and cargo loading . The result of this process is a lattice-like structure, beautifully revealed by Heuser's studies of the inner surface of the plasma membrane.

The large GTPase dynamin, which is recruited during coat assembly and then in a further burst just prior to vesicle budding , has an essential role in pinching the nascent coated vesicle from the plasma membrane. Interfering with dynamin function leads to disruption of clathrin-dependent endocytosis, but also to an impairment of other forms of trafficking . Two types of dominant interfering dynamin mutants have been prepared, in order to obtain either an acute or a long-term block in endocytosis. An acute block (in minutes) can be achieved by expressing a temperature-sensitive dynamin mutant (dynamin^{ts}) . A short-term shift from permissive (30°C) to non-permissive (38°C) temperature blocks receptor-mediated transferrin uptake in cells expressing dynamin^{ts}. A long-term endocytic block (days) can be obtained by over-expressing dynamin mutants (e.g., dynamin^{K44A}) defective in GTP binding and hydrolysis . Both forms of dynamin inactivation result in total inhibition of clathrin dynamics (Ehrlich and

Kirchhausen, in preparation) but in only incomplete blockage of transferrin uptake . Thus, it appears that transferrin can enter by alternative, although less efficient, routes. A continuing challenge, illustrated by the experiments in this paper, is to sort out, in any particular case, the relative importance of different traffic pathways.

During a long-term endocytic block, a cell responds in various ways: there is a return to normal levels of fluid phase uptake (which initially falls when endocytosis is inhibited) and there is an increase in endosomal pH sufficient to inhibit infection by pH-sensitive viruses .The H^+ pump is a component of clathrin-coated vesicles , and coated pit budding requires dynamin function . It is therefore plausible that the change in pH reflects a substantial decrease in the amount of H^+ pump delivered by endocytic-coated vesicles from the cell surface to endosomes. Thus, processes such as iron delivery by transferrin or virus translocation may depend on more than one kind of cargo delivered by the clathrin pathway, and distinguishing among alternative routes of uptake may require careful dissection of multiple effects.

Entry of anthrax toxin is a complex process of this kind. The toxicity of *Bacillus anthracis* requires the combined activity of three bacterial proteins, known as protective antigen (PA), edema factor (EF) and lethal factor (LF). PA is the carrier for EF and LF, facilitating their delivery to the cytosol of a target cell . EF is an adenylyl cyclase that catalyzes the synthesis of cyclic AMP , and LF is a

protease that cleaves mitogen-activated protein kinase kinase . These proteins are released from the bacteria as monomers and assemble into a complex upon binding to the anthrax toxin receptor (ATR) on the surface of the target cell . Before formation of this complex, however, the following steps need to occur. First, the full length PA (or PA(83)) must be cleaved at the cell surface by furin to form PA(63) which then assembles into a ring-shaped pre-pore heptamer $PA(63)_7$. The heptamer binds EF and LF and the ternary complex is internalized and transported to an endosome , where acidic conditions lead to formation of a $PA(63)_7$ pore for transfer of EF or LF into the cytosol .

What role does the clathrin pathway have in the uptake of anthrax toxin? A long-term block in endocytosis by expression of $dynamin^{K44A}$ leads to a retardation in the kinetics of the transition from PA(83) to the SDS-resistant $PA(63)_7$. From these and other biochemical results, as well as from morphological studies, it was concluded that anthrax toxin enters cells by a clathrin-dependent route. The considerations discussed above suggest an alternative possibility: like transferrin, the toxin might be able to take multiple routes to an endosome, and the observed decrease in $PA(63)_7$ conversion might reflect not just a lower rate of delivery to endosomes but also an elevated pH in that compartment.

We compare here the effects of acute and long-term impairment of dynamin function on the toxicity of LFn-DTA, a surrogate of LF. LFn-DTA is a fusion protein that contains the N-terminus of LF and the catalytic subunit of diphtheria

toxin. LFn-DTA binds as well as LF itself to $PA(63)_7$ heptamers, and it requires low pH to translocate from an endosome into the cytosol, where it leads quickly to inhibition of protein synthesis . Inhibition of cellular protein synthesis is thus a straightforward measure of LFn-DTA entry and translocation. We find that a short-term block in dynamin function results in a five-fold increase in the dose of LFn-DTA required for maximal toxicity, but not in a complete inhibition of toxin entry. We therefore suggest that LFn-DTA/$PA(63)_7$ can use more than one entry pathway. In contrast, a long-lasting block in dynamin function has the additional effect of conferring toxin resistance, similar to the resistance obtained by specific inhibition of the H^+ pump with bafilomycin A_1 . We conclude that perturbing the endocytic traffic of the proton pump may have a more profound effect on toxin entry than preventing toxin uptake by clathrin-coated vesicles.

MATERIALS AND METHODS

Reagents

HeLa cells expressing HA-tagged wild type dynamin1^{wt}, dynamin^{K44A} and the temperature-sensitive dynamin^{ts} under control of tetracycline (tet-off system) were kindly provided by S. Schmid (Scripps Institute, La Jolla, CA). LFn-DTA and PA were purified as described . The CHO-R1.1 cells not expressing the anthrax toxin receptor were obtained from J. Young (University of Wisconsin-Madison, Madison, Wi). The following reagents were obtained from commercial sources: Bafilomycin A_1 and tetracycline (Sigma Chemicals, St. Louis, MO); fetal bovine serum (Gemini, Calabasas, CA); penicillin/streptomycin, glutamine, and DMEM (Mediatech, Herndon, VA); leucine-free Ham's medium (Gibco BRL, Rockville, MD); L-[3,4,5-^{3}H]-leucine (New England Nuclear/Perkin Elmer, Boston, MA); and Alexa 488-transferrin and Alexa 594 goat anti-mouse antibodies (Molecular Probes, Eugene, OR).

Toxicity Assay

HeLa cells expressing dynamin^{wt} or dynamin^{K44A} were maintained at 37°C and 5% CO_2 in DMEM medium supplemented with 10% FBS, 100 U penicillin, 0.1 mg/ml penicillin/streptomycin, 2 mM glutamine and 1µg/ml tetracycline. The assays were done using 2x10^{4} cells plated in 24-well plates. Expression of dynamin was attained upon removal of tetracycline by extensive washes, and cells were grown for 2-5 days before experimentation at 37°C (dynamin^{wt}, dynamin^{K44A}) or 30°C (dynamin^{ts}). Every experiment carried as a control an

aliquot of cells kept in tetracycline. Experiments were initiated by replacing the medium with fresh DMEM containing 10^{-10} M LFn-DTA and different amounts of PA(83) at 37°C (dynamin^{wt}, dynamin^{K44A}) or 30°C and 38°C (dynamin^{ts}) . After 90 min of incubation, the media containing toxin were removed and replaced by leucine-free Ham's medium containing 1 µCi/ml ^{3}H-leucine for 1 hr at 37°C. Protein synthesis was determined by TCA precipitation and cell lysis ; a portion of the precipitate was used to normalize the data based on the determination total protein content. Data are presented as percentage of ^{3}H-leucine incorporation when using 10^{-11}M PA(83).

Transferrin Uptake

The effect of expressing the dynamin mutants on clathrin-mediated endocytosis was verified by fluorescence microscopy using fluorescently tagged Alexa 488-transferrin. HeLa cells plated in 12 mm coverslips placed in 24-well plates were transferred to KRH buffer (120mM NaCl, 6mM KCl, 1.2mM $MgCl_2$, 1mM $CaCl_2$, 25mM Hepes, pH 7.4; 1.8 mg glucose/ml, and 2 mg BSA/ml) for 30 min at the required temperature, followed by incubation for 20 min in KRH together with 500 nM Alexa 488-transferrin. The uptake was terminated by placing the 24-well plates on top of wet ice and washing the cells with ice-cold PBS, containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$. The cells were visualized by epifluorescence microscopy with a 63x lens after fixation with 3% paraformaldehyde, and stained with the monoclonal 12CA5 antibody specific for the HA-tagged and secondary Alexa 594 goat anti-mouse antibodies in the presence of 0.04% saponin. Cells

contained in many independent fields were scored for presence of absence of transferrin uptake.

RESULTS AND DISCUSSION

Toxicity assay

We first searched for incubation conditions that would lead to complete inhibition in Hela cells of protein synthesis by LFn-DTA and PA(83). We found that exposure of the Hela cells for 90 min to a mixture of 10^{-10} M LFn-DTA and 1-5 x 10^{-10} M PA(83) was sufficient to inhibit protein synthesis by 50%; we could elicit a complete block with 10^{-10} M LFn-DTA and 10^{-8} M PA(83) (Fig. 1A). Since the toxic effect requires expression of ATR (Fig. 1B), we can rule out the possibility that entry of LFn-DTA and PA(83) follows a non-specific, fluid-phase uptake route. The toxic effect of LFn-DTA requires exposure of the LFn-DTA/$PA(63)_7$ complex to the acidic environment encountered in endosomes . We used the H^+-ATPase inhibitor bafilomycin A_1 to increase the endosomal pH and thus verify that the toxic effect of LFn-DTA in our experiments was also pH-dependent. The increase in endosomal pH was confirmed by fluorescence microscopy following the pH-sensitive fluorescence of Lyso-Tracker within lysosomes (not shown). As shown in Fig. 1A treatment with bafilomycin A_1 completely blocked LFn-DTA toxicity, even when the amount of PA(83) used exceeded by 100 fold the quantity required under normal conditions for a maximum toxic response. Although exposure to bafilomycin A_1 does not prevent clathrin-mediated endocytosis, we added LFn-DTA and PA(83) 30 min prior to exposure to bafilomycin A_1 to ensure LFn-DTA entry. We conclude from these observations that the toxicity assay based on LFn-DTA is a valid probe for cell entry and access to endosomes.

Effect of acute block of dynamin function on the toxic activity of LFn-DTA

We induced the expression of dynamin^{ts} for three days in Hela cells maintained at the permissive temperature of 30°C and used fluorescence microscopy to confirm previous work showing that, within minutes of transfer to the non-permissive temperature of 38°C, at least 90% of the Hela cells have a marked reduction in receptor-mediated endocytosis of transferrin (Fig 2A). Under these conditions of acute block in dynamin function and clathrin-mediated uptake we found a small but reproducible increase of about 5-6 fold in the amount of PA(83) required for 50% inhibition of protein synthesis by LFn-DTA (Fig 2B). This observation is consistent with reduction in the efficiency of LFn-DTA delivery, since exposure to higher concentrations of PA(83) still led to complete inhibition of protein synthesis. At least 90% of the cells had a block in clathrin-based endocytosis of transferrin (99 out of 120 cells counted) and hence also, we assume, in clathrin-mediated uptake of LFn-DTA, which nonetheless clearly entered these cells. We therefore suggest that, like transferrin, LFn-DTA enters through one or more alternative route(s). It is known that under similar conditions of complete blockage of clathrin-coated pit budding, the rate of transferrin uptake is reduced about four-fold although it still can access endosomes .

Effect of long-lasting block of dynamin function on the toxic activity of LFn-DTA

We then used long-term expression of dynamin^{K44A} to interfere with dynamin function and block clathrin-mediated uptake. We used fluorescence microscopy

to confirm previous work showing that two days after the removal of tetracycline more than 85% of the Hela cells expressed dynamin^{K44A}, resulting in a marked reduction in receptor-mediated endocytosis of transferrin (Fig 3A, panel c). In contrast, the uptake of transferrin in control cells expressing dynamin^{wt} (panel a) was indistinguishable from the uptake in cells only expressing endogenous dynamin (panels b and d). Reduction in LFn-DTA toxicity under long-lasting block of dynamin function (Fig. 3B, full circle) showed two types of effects. One effect was similar to the one observed upon acute block of dynamin function and corresponded to the same 5-6 fold increase in PA(83) concentration required to elicit a 50% inhibition in protein synthesis. The second effect was a failure to block proteins synthesis by more than 50%, even at concentrations of PA(83) as high as 10^{-6} M, that is 100-fold higher than the amount required for complete blockage in normal cells . Others have observed an increase in endosomal pH after long-term blockage of endocytosis by overexpression of dynamin^{K44A}. We suggest that the failure of LFn-DTA to block protein synthesis completely reflects interference of LFn-DTA translocation from endosomes to the cytosol in a corresponding proportion of the cells, due to elevated endosomal pH. Any cell with suitable elevated endosomal pH will fail to transloctate internalized LFn-DTA and about half the cells in our experiments appear to have been in that category.

Finally we expressed dynamin^{ts} for 5 days at the permissive temperature of 30°C before a brief transfer to 38°C (Fig 4A). We noticed a small decrease of transferrin uptake (measured at 30°C) in cells expressing dynamin^{ts} for 5-days at

30°C when compared to cells kept at the same conditions but not expressing dynamin^{ts} (compare panels a and b, Fig. 4A). These results suggest that dynamin^{ts}, might have reduced function even at the nominally permissive temperature. Nevertheless, shift to 38°C elicited a strong block in transferrin uptake, essentially the same as detected in cells expressing dynamin^{ts} for three instead of five days (compare panels c in Fig. 2 and 4). By contrast, the effect of temperature shift in LFn-DTA toxicity resembled what we found with cells expressing dynamin^{K44A} rather than the simpler effect seen in cells expressing dynamin^{ts} for only three days (compare full circle tracings in Fig. 3B and 4B). That is, extended expression of dynamin^{ts} appears to have prevented about half LFn-DTA translocation in the cells, presumably by elevating endosomal pH.

Conclusion

Our results indicate that a major route for LFn-DTA entry depends on the normal function of dynamin. However, a complete block in the clathrin-dependent mechanism is not sufficient to prevent LFn-DTA toxicity. Thus, other endocytic pathway(s) can also be used by LFn-DTA to reach an acidic endosomal compartment. Low pH is essential for the formation of the $PA(63)_7$ pore and for the translocation of bound LFn-DTA into the cytosol. The contents of most membrane-bound organelles are under tight control, in part due to intracellular vesicular traffic. Interference with clathrin-coated vesicle traffic by inhibition of coated pit budding results in a marked increase in endosomal pH . This pH increase, and not the reduction in endocytosis, probably accounts for the marked

protection against the toxic effects of LFn-DTA observed after long-term expression of dynamin^{ts} or dynamin^{K44A}.

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I have fond remembrances off my association with John Heuser. I warmly recall when, on a summer morning, I entered John's studio in Martha's Vineyard to share his beautiful and puzzling images of clathrin lattices decorating the inner surface of cells. We then spent hours looking at images of clathrin triskelions, trying to understand their structure. Many years later, I am still driven by the power and the intellectual stimulation provided by those images, and many of the experiments we perform today have very close connections to our earlier discussions.

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FIGURE LEGENDS

Figure. 1. Toxicity assay. (A) Inhibition of LFn-DTA toxicity by Bafilomycin A_1. After a 30 min pre-incubation of wild-type Hela cells with DMSO and/or Bafilomycin A_1, the cells were incubated for another 90 min with $1x10^{-10}$ M LFn-DTA and different amounts of PA(83) in the presence of 0.66% DMSO (black squares), 0.66% DMSO and 200 nM Bafilomycin A_1 (black circle) or left as a control (white circles). **(B)** ATR is required for LFn-DTA toxicity. CHO-R1.1 cells defective in ATR (black circles) and wild type CHO-R1 (white circles) cells were incubated for 90 min with $1x10^{-10}$ M LFn-DTA and different amounts of PA(83). At the end of the incubation period with LFn-DTA and PA(83), the medium was removed and replaced with leucine-free HAM-F12 supplemented with ^{3}H-leucine for 60 min at 37°C for 1 hr. The cells were then washed with ice-cold PBS followed by ice-cold 5% TCA. The amount of ^{3}H-leucine incorporated in the TCA precipitate is a measure of protein synthesis. The data represents an average of 2 experiments.

Figure 2. Inhibition of LFn-DTA toxicity by acute block of dynamin function. (A) Acute block of dynamin function prevents the receptor-mediated endocytosis of transferrin. Hela cells were grown for 72 hr at 30°C in the absence or presence of tetracycline to express (+dyn^{ts}) or not (-dyn^{ts}) the temperature sensitive mutant $dynamin^{ts}$. The temperature of the cells was then either kept at 30°C or shifted to 38°C for 20 min, followed by incubation with Alexa 488-transferrin (green) for another 20 min. The appearance of the intracellular

punctuate pattern is characteristic of transferrin internalized into the endosomal compartment. Expression of HA-tagged dynamin^{ts} (red) was determined after fixation and antibody staining. About 90% of the cells expressed dynamin^{K44A} as judged by HA-staining and by the number of cells whose transferrin uptake was blocked.

(B) Acute block of dynamin function reduces the susceptibility to inhibition of protein synthesis by LFn-DTA. The toxicity assay was performed as described in Fig. 1, using Hela cells expressing (black circles and squares) or not (white circles and squares) dynamin^{ts}. Block of dynamin function was initiated by shifting the temperature to 38°C for 20 min, followed by addition of LFn-DTA and PA(83) for another 90 min. At the end of this period, the cells were transferred to media containing ^{3}H-leucine for one hour and processed for the protein synthesis assay. The data represent the average of 5 independent experiments carried out at the permissive temperature of 30°C (squares) and the non-permissive temperature of 38°C (circles).

Fig. 3. Inhibition of LFn-DTA toxicity by long-term block of dynamin function by expression of dynamin^{K44A}. (A) Long-term block of dynamin function by expression of dynamin^{K44A} prevents the receptor-mediated endocytosis of transferrin. Hela cells were grown for 48 hr at 37°C in the absence or presence of tetracycline and allowed to express (+dyn^{K44A}) or not (-dyn^{K44A}) the dominant mutant dynamin^{K44A}. Cells expressing (+dyn^{wt}) or not (-dyn^{wt}) wild type dynamin were used as controls. The cells where incubated with Alexa 488-

transferrin (green) for 20 min. Expression of HA-tagged dynamin^{K44A} and dynamin^{wt} (red) was determined after fixation and antibody staining. About 85% of the cells expressed dynamin^{K44A} as judged by HA-staining and by the number of cells whose transferrin uptake was blocked.

(B) Long-term block of dynamin function reduces the susceptibility to inhibition of protein synthesis by LFn-DTA. The toxicity assay was performed as described in Fig. 1, using Hela cells expressing (black circles) or not (white circles) dynamin^{K44A} and Hela cells expressing (black square) or not (white square) wild-type dynamin (dynamin^{wt}) for 48 hr. The data represent the average of 3 independent experiments.

Figure 4. Effect of prolonged expression of dynamin^{ts} followed by Inhibition by acute block in its function on LFn-DTA toxicity. (A) Acute block of dynamin function prevents the receptor-mediated endocytosis of transferrin. Hela cells were grown for 120 hr at 30°C in the absence or presence of tetracycline and allowed to express (+dyn^{ts}) or not (-dyn^{ts}) the temperature sensitive dynamin^{ts}. The temperature of the cells was kept at 30°C or shifted to 38°C for 20 min, followed by incubation with Alexa 488-transferrin (green) for another 20 min. About 90% of the cells expressed dynamin^{ts} as judged by HA-staining and by the number of cells whose transferrin uptake was blocked. The coarse and perinuclear clusters of dynamin^{ts} in panel c, usually observed after 5 days of expression and shift to 38°C, were also seen in HeLa cells expressing dynamin^{K44A}.

(B) Acute block of dynamin function reduces the susceptibility to inhibition of protein synthesis by LFn-DTA. The toxicity assay was performed as described in Fig. 1, using Hela cells expressing (black circles and squares) or not (white circles and squares) dynamin^{ts}. Block of dynamin function was initiated by shifting the temperature to 38°C for 20 min and followed by addition of LFn-DTA and variable amounts of PA(83) for another 90 min. The data represent the average of 3 independent experiments carried out at the permissive temperature of 30°C (squares) and the non-permissive temperature of 38°C (circles).

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