Receptor-specific requirements for anthrax toxin delivery into cells

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The three proteins that constitute anthrax toxin self-assemble into toxic complexes after one of these proteins, protective antigen (PA), binds to tumor endothelial marker 8 (TEM8) or capillary morphogenesis protein 2 (CMG2) cellular receptors. The toxin receptor complexes are internalized, and acidic endosomal pH triggers pore formation by PA and translocation of the catalytic subunits into the cytosol. In this study we show that the pH threshold for conversion of the PA prepore to the pore and for translocation differs by approximately a pH unit, depending on whether the TEM8 or CMG2 receptor is used. For TEM8-associated toxin, these events can occur at close to neutral pH values, and they show relatively low sensitivity to ammonium chloride treatment in cells. In contrast, with CMG2-associated toxin, these events require more acidic conditions and are highly sensitive to ammonium chloride. We show, furthermore, that PA dissociates from TEM8 and CMG2 upon pore formation. Our results are consistent with a model in which translocation depends on pore formation and pore formation, in turn, depends on release of PA from its receptor. We propose that because PA binds to CMG2 with much higher affinity than it does to TEM8, a lower pH is needed to attenuate CMG2 binding to allow pore formation. Our results suggest that toxin can form pores at different points in the endocytic pathway, depending on which receptor is used for entry.

capillary morphogenesis protein 2 | tumor endothelial marker 8 | toxin entry

Bacillus anthracis, the causative agent of anthrax, secretes a toxin that is believed to be instrumental in causing anthrax disease symptoms leading to death. Anthrax toxin consists of three proteins, protective antigen (PA), which is a receptor-binding and pore-forming subunit; lethal factor (LF), which is a protease that cleaves mitogen-activated protein kinase kinase family members; and edema factor (EF), which is an adenylate cyclase that raises cAMP levels in cells (1). PA is synthesized as an 83-kDa protein (PA_{83}) for which two cell surface receptors have been identified: tumor endothelial marker 8 (TEM8) (2, 3) and capillary morphogenesis protein 2 (CMG2) (4). Two splice variant mRNAs derived from the TEM8 gene (sv1 and sv2) encode functional anthrax toxin receptors (2, 5). TEM8 expression has been documented in epithelium of the lung, intestine, and skin, the three routes of entry in anthrax infection (6). The CMG2 gene, which has been shown to be broadly expressed in different tissues (4), encodes three protein isoforms, two of which, $CMG2^{488}$ and $CMG2^{489}$, are anthrax toxin receptors (4) (H.M.S., unpublished data).

Receptor-bound PA_{83} is cleaved by a cellular protease to generate a 20-kDa PA_{20} subunit and a 63-kDa subunit (PA_{63}). The larger subunit assembles into the heptameric $(PA_{63})_7$ prepore in lipid rafts (7–9). EF and LF bind to the prepore, and the toxin–receptor complexes are internalized by clathrin-dependent endocytosis and by other endocytic mechanisms (9, 10). These complexes are then exposed to increasingly acidic environments as they are trafficked from early/sorting endosomes to late endosomes. The current model of anthrax toxin entry invokes $(PA_{63})_7$ prepore conversion to a 14-stranded β-barrel-containing pore on the membranes of intraluminal vesicles that are formed within mildly acidic early/sorting endosomes (7, 11–16) (Fig. 1*a*). LF and EF are then believed to translocate into the luminal compartment of these vesicles, which is topologically equivalent to the cytosol. These vesicles then traffic to late endosomes, where they back-fuse with endosomal-limiting membranes to release LF and EF into the cytosol (11).

The PA protein consists of four distinct domains, which have each been assigned different functions (1). Domain 1 is the site of proteolytic processing that gives rise to PA_{20} and PA_{63}. The portion of domain 1 that is associated with PA_{63} binds EF and LF after PA_{63} oligomerization (8). Domain 2 contributes to receptor binding and undergoes low pH-induced structural rearrangements to form the membrane-associated pore (17–19). Domain 3 is involved in PA_{63} oligomerization (20). Domain 4 is primarily involved in receptor binding (21, 22).

X-ray structural studies of complexes consisting of the toxin-binding, integrin-like I domain of CMG2 bound to PA_{83} or to the $(PA_{63})_7$ prepore have revealed how CMG2 binds to PA domains 2 and 4 (17, 18). Based on this model, it has been proposed that low pH causes the release of receptor from domain 2 to facilitate pore formation (17, 18). Consistent with this idea, binding of soluble CMG2 I domain to the $(PA_{63})_7$ prepore shifts the threshold of the conformational prepore-to-pore transition to a more acidic pH range (17). It has further been proposed that the receptor might remain bound, presumably to domain 4 of PA, so that it serves as a structural support for the newly formed pore (18, 23).

There has been speculation that destabilization of the folding of EF and LF by low pH could foster translocation through the newly formed $(PA_{63})_7$ pore (1, 24–26) (Fig. 1*a*). The N-terminal PA-binding regions of EF and LF partially unfold at the pH 5–6 range, generating a molten globule state of these proteins (25), and translocation of LF_N (the N-terminal PA-binding region of LF) through $(PA_{63})_7$ pores that are formed on cell surfaces requires pH values of $\approx$5.5 or less (26).

Before this work, it was believed that characteristics of the toxin entry mechanism were the same regardless of cell type or receptor usage. Here we show that receptor type has a profound impact on the pH threshold required for pore formation and translocation of anthrax toxin into cells. We also provide evidence that receptor dissociation is linked to pore formation and

Abbreviations: TEM8, tumor endothelial marker 8; CMG2, capillary morphogenesis protein 2; PA, protective antigen; LF, lethal factor; EF, edema factor; LF_N-DTA, lethal factor N-terminal diphtheria toxin A chain; MEK, mitogen activated protein kinase; CHO, Chinese hamster ovary.

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Fig. 1. Pore formation in cells expressing TEM8 does not require low pH. (*a*) Existing model of anthrax lethal toxin internalization and pore formation. The receptor–toxin complex is internalized and trafficked to an acidic endosome. Endosomal low pH triggers $(PA_{63})_7$ pore formation and partial unfolding of the toxin catalytic subunits before translocation through the pore into the cytosolic compartment. (*b*) Different cell types were exposed to lethal toxin (PA plus LF) in the presence or absence of ammonium chloride. The cells were lysed and subjected to SDS/PAGE and immunoblotting with anti-PA antibody. The location of the SDS-resistant $(PA_{63})_7$ heptamer is indicated by an arrow. (*c*) Pyrene-labeled N306C PA was incubated with soluble CMG2 (sCMG2), soluble TEM8 (sTEM8), or without receptors in buffers at different pH values, and the resulting pyrene excimer fluorescence levels were measured. (*d*) PA_{63} was bound to dGAB-treated cells for 2 h at 4°C, unbound PA was removed by washing, and samples were incubated at 37°C for 10 min at the indicated pH in the presence of dGAB. Cells were then lysed, and protein lysates were subjected to SDS/PAGE and immunoblotting with an anti-PA antibody.

show that LF translocation can occur under near-neutral pH conditions when the toxin is bound to TEM8.

Methods

Reagents. PA_{83}, PA_{63}, LF, and anti-PA goat serum were obtained from ListLabs. LF_N-diphtheria toxin A chain (LF_N-DTA) was produced as described in ref. 27. Anti-PA rabbit serum was a gift from Ken Bradley (University of California, Los Angeles). Anti-rabbit-horseradish peroxidase was obtained from Amersham Pharmacia. Anti-MEK1 (MEK, mitogen activated protein kinase) N-terminal was obtained from Upstate Biotechnology (Lake Placid, NY), the C-terminal antibody from Santa Cruz Biotechnology, and anti-EGFP was obtained from Covance (Berkeley, CA). Soluble CMG2 was the S38 protein (28), and soluble TEM8 (residues 1–234) was produced in the 293 FreeStyle expression system according to the manufacturer's instructions (Invitrogen) and purified by Ni-nitrilotriacetic acid and gel filtration.

Cell Culture. Chinese hamster ovary (CHO) cells were maintained in F12 medium with GlutaMAX (Invitrogen) supplemented with 10% calf serum (Invitrogen) and 1× pennicillin/streptomycin (Invitrogen). RAW 264.7 and HeLa cells were maintained in DMEM (Invitrogen) supplemented with 10% FBS (HyClone) and 1× pennicillin/streptomycin (Invitrogen). For the cells incubated with NH_4Cl and dGAB (see below), these reagents were present throughout each experiment.

SDS-Resistant Pore Formation. Cells were pretreated with or without 30 mM NH_4Cl in supplemented F12 medium for 1 h at 37°C. Cells were incubated with 10^{-8} M PA_{83} and 10^{-9} M LF for 1 h at 37°C. Cells were then washed with PBS, trypsinized, and neutralized with supplemented F12 medium. Cells were then washed twice with PBS and lysed in TBS-containing 1% Nonidet P-40 with Complete EDTA-free protease inhibitor mixture (Roche Applied Science, Indianapolis). Lysates were pelleted, and supernatants were adjusted to 2% SDS in reducing gel sample buffer and subjected to SDS/PAGE on 3–8% acrylamide Tris-acetate gels (Invitrogen) without boiling. Proteins were transferred to polyvinylidene difluoride, and PA was detected by immunoblotting with anti-PA, anti-rabbit-horseradish peroxidase, and SuperSignal femto Western detection reagent (Pierce).

To assay cell-surface SDS-resistant pore formation, cells were pretreated with 50 mM 2-deoxy-glucose (Sigma)/10 mM sodium azide (Sigma)/200 nM bafilomycin A1 (Alexis Biochemicals) (dGAB) in supplemented F12 medium for 45 min at 37°C. Cells were incubated with 10^{-8} M PA_{63} for 2 h at 4°C, washed with PBS, and shifted to 37°C for 10 min in the following buffers diluted into PBS: 50 mM Tris, pH 8; 50 mM HEPES, pH 7.3 or 6.8; and 50 mM MES pH 6.4, 6, 5.5, or 5.2. Cells were then washed in cold PBS, harvested, and lysed, and 20-μg protein samples were subjected to SDS/PAGE and immunoblotting as described above.

Immunoprecipitation. Pores were formed at the cell surface in the presence of dGAB as described above. Cells were washed twice with cold PBS, harvested and lysed on ice as described above except that a 1:500 dilution of an anti-PA rabbit serum was included at all harvest steps. The samples were then subjected to centrifugation at 15,000 × g for 10 min, and the supernatant was incubated at 4°C for 1 h with rabbit anti-PA antibody (1:500 dilution) and then with protein A-Sepharose beads for an additional hour. The beads were then washed and suspended in a gel sample buffer containing 2% SDS. Samples were resolved on 3–8% Tris-acetate or 8% Tris-glycine gels and transferred to polyvinylidene difluoride and probed with anti-PA goat serum or an anti-EGFP mouse monoclonal antibody.

Pyrene Fluorescence. N306C PA_{63} was produced and labeled with pyrene as described in ref. 17. Labeled $(PA_{63})_7$ prepore was mixed with soluble CMG2 or soluble TEM8 as described in ref. 17, except that 1 mM $MgCl_2$ was included in the mixture. pH was adjusted to the indicated values by using 0.1 equivalents of either 1 M Tris, pH 8; 1 M HEPES, pH 7; 1 M BisTris, pH6; or 1 M sodium acetate, pH 5. After 30 min, samples were analyzed in a 1- × 0.5-cm quartz cuvette using an ISS (Champaign, Illinois) fluorometer at an excitation wavelength of 341 nm. Emission was measured from 360 to 600 nm and normalized against the maximal fluorescence at 384 nm.

Intoxication Assays. For LF_N-DTA intoxication and MEK1 cleavage assays, cells were pretreated with or without 30 mM NH_4Cl in supplemented F12 medium for 1 h at 37°C. In the LF_N-DTA experiments, cells were incubated with the indicated concentrations of PA_{83} and 10^{-9} M LF_N-DTA for 2 h at 37°C. The medium was then removed and replaced with fresh medium, and the cells were incubated for an additional 2 h at 37°C. The medium was then replaced with DMEM without glutamine, leucine, and sodium pyruvate (MP Biomedicals, Irvine, CA) supplemented with 2 mM L-glutamine (Invitrogen), 0.45 mM sodium pyruvate (Sigma), and 1 μCi/ml, 173 Ci/mmol [^{3}H]leucine (1 Ci = 37 GBq; PerkinElmer). Cells were incubated at 37°C for a further 2 h, washed three times with cold PBS, resuspended in EcoLume scintillation fluid (MP Biomedicals), and assayed with a Top-Count NXT microplate scintillation counter (Packard).

In the MEK1 cleavage assays cells were incubated with 2.5 × 10^{-8} M PA_{83} and 5 × 10^{-9} M LF for 2 h at 4°C, washed, and shifted to 37°C for the indicated times. In the case of the 4-h time point, the medium was exchanged with fresh NH_4Cl-containing medium after 2 h had elapsed. Cells were lysed in TBS-containing 1% Nonidet P-40 as described above, and 12-mg protein samples were resolved by 8% acrylamide Tris-glycine SDS/PAGE, transferred to a polyvinylidene difluoride membrane, and detected with anti-MEK1 N-terminal and C-terminal antibodies, followed by anti-rabbit-horseradish peroxidase secondary antibodies and SuperSignal femto Western detection reagent (Pierce).

Results

To determine whether the TEM8 and CMG2 receptors influence the triggering mechanism that leads to anthrax toxin pore formation, we investigated the pH thresholds for this process in PA receptor-deficient CHO-R1.1 cells (2) engineered to express either receptor (Fig. 5, which is published as supporting information on the PNAS web site). PA_{83} and LF were added to the cells in the presence of ammonium chloride, a lysosomotropic agent that elevates the pH of acidic endosomes to approximately pH 6.5 or higher (29, 30). Pore formation was monitored by the conversion of PA to an SDS-resistant oligomeric form (7, 16) (Fig. 1 *a* and *b*). Consistent with a low pH requirement, the ammonium chloride treatment blocked $(PA_{63})_7$ pore formation in the CMG2-expressing cells and in CHO-K1 cells and RAW 264.7 mouse macrophage cells that express endogenous anthrax toxin receptors (Fig. 1*b*). By contrast, a significant amount of $(PA_{63})_7$ pore formation occurred with the TEM8-expressing cells and with HeLa cells in the presence of the lysosomotropic agent (Fig. 1*b*). These data indicate that pore formation can occur at near-neutral pH when the toxin is bound to TEM8 but not when it is bound to CMG2. Furthermore, these results revealed an unexpected difference between three cell types (HeLa, CHO-K1, and RAW 264.7 cells) that were previously used to derive a unified model for anthrax toxin entry (9, 11, 15).

To define the pH threshold of TEM8-associated $(PA_{63})_7$ pore formation, we used a pyrene-labeled form of PA (N306C PA) that generates an excimer fluorescent signal upon pore formation (16). Pyrene-labeled N306C $(PA_{63})_7$ prepore was bound to soluble forms of TEM8 or CMG2 (28) *in vitro* in conditions under which the toxin subunit was fully occupied by soluble receptors, as judged by native PAGE (Fig. 6, which is published as supporting information on the PNAS web site). The pH was adjusted to various values, and the resulting excimer fluorescence was monitored. When bound to TEM8, $(PA_{63})_7$ pore formation occurred at pH values ranging from pH 6.8 to 7.1, but, when bound to CMG2, it occurred at pH values between pH 5.7 and 5.8 (Fig. 1*c*). As noted previously (17), PA formed pores between pH 7.1 and 7.5 when no soluble receptor was present (Fig. 1*c*).

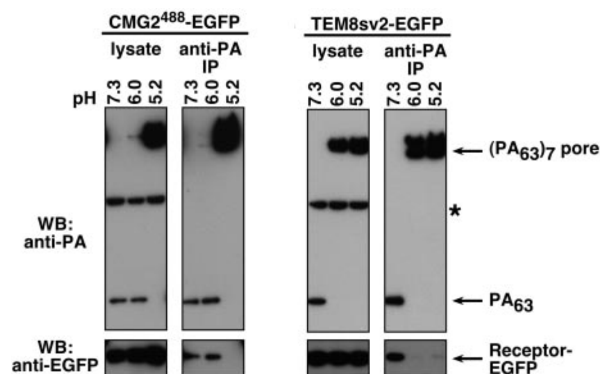

Fig. 2. Toxin dissociates from receptor upon pore formation. PA_{63} was bound to dGAB-treated cells as described in Fig. 1*d*. Cells were treated at the indicated pH values for 10 min and lysed in the presence of an anti-PA rabbit antiserum that does not block PA receptor binding (data not shown). Lysates were resolved directly or immunoprecipitated with anti-PA rabbit serum and immunoblotted with an anti-PA goat serum or an anti-EGFP antibody to detect receptor proteins. IP, immunoprecipitation; WB, Western blot; *, nonspecific cellular protein detected by goat anti-PA.

To examine the pH threshold of pore formation when PA is bound to cell membrane-associated receptors, PA_{63} was added to cells in the presence of a mixture of 2-deoxyglucose, sodium azide, and bafilomycin A1 (dGAB) (31). The dGAB treatment prevented toxin-receptor complex internalization (Fig. 7, which is published as supporting information on the PNAS web site). This experiment confirmed that $(PA_{63})_7$ pores formed under mildly acidic pH conditions (pH 6.4) when PA was bound to TEM8 but at more highly acidic conditions (pH 5.2) when it was bound to CMG2 (Fig. 1*d*). The difference between these pH threshold measurements and those obtained with pyrene-labeled N306C PA may be due to the effect of cell membranes on the pH requirements for $(PA_{63})_7$ pore formation.

To determine whether receptors remain bound during $(PA_{63})_7$ pore formation, PA_{63} was bound to dGAB-treated cells that expressed EGFP-tagged versions of $CMG2^{488}$ or TEM8 sv2. The cells were then incubated in buffers at pH 7.3, 6, or 5.2. The cells were then lysed, and PA-associated receptors were detected by immunoprecipitation with an anti-PA antibody followed by immunobloting with an EGFP-specific antibody. The anti-PA antibody used in this study does not disrupt the PA-receptor interaction (data not shown). PA was associated with $CMG2^{488}$–EGFP in the absence of pore formation (pH 7.3 and pH 6) but not at pH 5.2 when pore formation was observed (Fig. 2 *Left*). Similarly, PA was dissociated from TEM8sv2–EGFP under conditions of pore formation (pH 6 and pH 5.2) (Fig. 2 *Right*). These data indicate that the dissociation of PA from receptor is associated with $(PA_{63})_7$ pore formation.

To determine whether anthrax toxin can be translocated into cells under the mildly acidic conditions that are associated with TEM8-dependent $(PA_{63})_7$ pore formation, the entry of LF_N-DTA and LF into cells was monitored in the presence of ammonium chloride. LF_N-DTA entry was scored as inhibition of

Fig. 3. Ammonium chloride blocks toxin translocation into CMG2-expressing cells but not TEM8-expressing cells. (*a–g*) Cells were incubated with PA plus LF_N-DTA and metabolically labeled with [^{3}H]leucine to monitor protein synthesis in the presence (■) or absence (□) of NH_4Cl. The amount of radiolabeled protein was determined by scintillation counting and graphed from triplicate values ± SEM. (*h*) Cells were incubated with lethal toxin in the presence or absence of NH_4Cl for the indicated times. Cells were lysed, and protein lysates were subjected to SDS/PAGE and to immunoblotting with anti-MEK1 antibodies. NT, N-terminal; CT, C-terminal. Because only eight amino acids are removed from the N terminus of MEK1 by LF cleavage (32, 33), this does not significantly change the molecular weight of the cleaved MEK1 protein that is detected by the C-terminal antibody.

protein synthesis using a [^{3}H]leucine incorporation assay (27), and LF entry was scored by immunoblotting to detect the N-terminal cleavage of the MEK1 substrate (32, 33). Consistent with a requirement for an acidic pH, ammonium chloride treatment inhibited intoxication of the CMG2-expressing CHO-R1.1 cells, CHO-K1 cells, and RAW 264.7 cells (Fig. 3 *b–d* and *h*). By contrast, toxin was able to access the cytosol of TEM8-expressing CHO-R1.1 cells and HeLa cells in the presence of ammonium chloride (Fig. 3 *e–h*), although LF cleavage of the MEK1 substrate was delayed under these conditions (Fig. 3*h*). These data demonstrate that toxin translocation can also occur under the mildly acidic conditions that are associated with TEM8-dependent $(PA_{63})_7$ pore formation.

Discussion

The results of this study provide important insights into the role of the receptor in anthrax toxin entry. Specifically, anthrax toxin pore formation and translocation were found to occur under strikingly different acidic conditions when PA was bound to TEM8 instead of CMG2. Complete receptor dissociation was also implicated as being an integral component of the toxin pore-forming mechanism, and the toxin translocated into TEM8-expressing cells at near neutral pH (Fig. 4).

The finding that receptor type dictates different pH thresholds for anthrax toxin pore formation and translocation was unexpected. Previously, it was shown that treatment of peritoneal mouse macrophages and CHO-K1 cells with ammonium chloride, a weak base that neutralizes the pH of endosomes, prevents pore formation and translocation (7, 13). This result holds true for the PA receptor-deficient cells that were engineered to express CMG2, and it holds true for the RAW 264.7 mouse macrophage cells that were tested in this study. However, this finding does not hold true for the cells that were engineered to express TEM8 or for HeLa cells. Presumably, the distinct toxin behavior in RAW 264.7 and HeLa cells is due to differences in CMG2 versus TEM8 expression; however, this remains to be shown directly.

Previously, it was hypothesized that release of PA domain 2 from the receptor by the protonation of key histidine residues that are located at the PA-receptor binding interface triggers pore formation (17, 18). It was further postulated that the receptor remains bound to PA domain 4, perhaps to provide a structural support for the stalk that comprises the pore (18, 23). Because the coimmunoprecipitation experiments that we performed failed to detect receptors associated with the newly formed PA pores, our data instead favor a model in which complete receptor release is required for toxin pore formation. Indeed, this model is compatible with prior observations that demonstrated that functional $(PA_{63})_7$ pores could be formed in membranes lacking anthrax toxin receptors (12).

Previously, various lines of evidence obtained by studying anthrax toxin subunits in solution, on artificial lipid membranes, or on cell surfaces had indicated that the acidic pH characteristic of late endosomal compartments might be required for unfolding

Fig. 4. Model of the distinct receptor-specific pH thresholds for anthrax toxin pore formation and translocation. Receptor–toxin complexes are internalized and trafficked through the endosomal pathway. Pore formation and translocation occur at different pH thresholds for TEM8- versus CMG2-associated toxin, indicative of different endosomal routes of entry. Pore formation is associated with receptor dissociation. See Fig. 1 for a key to the symbols.

EF and LF before translocation through the $(PA_{63})_7$ pore (1, 24–26). By studying the translocation process within cellular endosomes, we show that LF_N-DTA and LF can translocate into the cytosol in TEM8-expressing cells that were incubated with ammonium chloride to raise endosomal pH. However, ammonium chloride treatment did slow the kinetics of LF-mediated cleavage of the MEK1 substrate in these cells (Fig. 3*h*). This effect may be due to less efficient pore formation (Fig. 1*b*) or LF unfolding (25) under more neutral pH conditions; or instead might be due to a reduction in the accessibility of MEK1, which might be concentrated on scaffolds on late endosomes (34), under these conditions. Our results are consistent with different receptor-specific pH requirements for pore formation in the endosomal pathway. Consistent with a previous report that PA forms pores in multivesicular regions of early/sorting endosomes (11), PA bound to TEM8 is predicted to form pores in the early/sorting endosome, where the pH is >6 (35). PA bound to CMG2, on the other hand, requires a much more acidic pH for pore formation (pH 5.2–5.5), one that is more characteristic of late endosomes (35). Based on these properties we propose that the receptor type might dictate the site of toxin pore formation in the endosomal pathway (Fig. 4).

It is likely that the interaction between anthrax toxin and multiple cell types contributes to anthrax pathogenesis. In the initial stages of anthrax infection, toxin plays a role in facilitating spore germination (36–38). Once the vegetative infection is established, many cells are exposed to the toxin. Previous studies have proposed a role for toxin in dendritic cells (39), vascular endothelial cells (40–42), and macrophages (43–47). In most of these cell types, the receptor repertoire is not known. However, because CMG2 and TEM8 appear to be broadly expressed in different cell types (4, 6), it will be important to establish whether heptameric PA-CMG2/TEM8 heterocomplexes can be formed and, if so, to determine the pH values of pore formation that are associated with heterocomplexes containing different ratios of the two receptors. Further investigation is also necessary to directly establish the endosomal sites of toxin entry into cells expressing TEM8 and CMG2 and to determine the effect of substrate subcellular localization on the efficiency of LF and EF action.

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