Receptor palmitoylation and ubiquitination regulate anthrax toxin endocytosis

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The anthrax toxin is composed of three independent polypeptide chains. Successful intoxication only occurs when heptamerization of the receptor-binding polypeptide, the protective antigen (PA), allows binding of the two enzymatic subunits before endocytosis. We show that this tailored behavior is caused by two counteracting posttranslational modifications in the cytoplasmic tail of PA receptors. The receptor is palmitoylated, and this unexpectedly prevents its association with lipid rafts and, thus, its premature ubiquitination. This second modification, which is mediated by the E3 ubiquitin ligase Cbl, only occurs in rafts and is required for rapid endocytosis of the receptor. As a consequence, cells expressing palmitoylation-defective mutant receptors are less sensitive to anthrax toxin because of a lower number of surface receptors as well as premature internalization of PA without a requirement for heptamerization.

Introduction

Anthrax toxin, one of the two major virulence factors produced by *Bacillus anthracis*, is composed of three independent polypeptide chains: the protective antigen (PA), which is involved in target cell binding; the edema factor (EF), a calmodulin-dependent adenylate cyclase; and the lethal factor (LF), a zinc-dependent metalloprotease (for reviews see Collier and Young, 2003; Abrami et al., 2005; Scobie and Young, 2005). Only PA is able to bind to target cells; thus, EF and LF must always act in binary combination with PA to be transported to the target cell cytosol, where they exert their activities. The two identified PA receptors, tumor endothelial marker 8 (TEM8) and capillary morphogenesis gene 2 (CMG2), are type I transmembrane proteins sharing ∼60% homology in their extracellular von Willebrand factor A domains and 68% identity in the first 145 residues of their cytoplasmic tails (Scobie and Young, 2005).

A relatively clear view of the mode of action of anthrax toxin has emerged over recent years (for reviews see Collier and Young, 2003; Abrami et al., 2005; Scobie and Young, 2005). PA is produced as an 83-kD protein that is unable to interact with EF and LF. At the target cell surface, proteolytic processing of PA83 leads to PA63, which remains receptor bound and can polymerize into a heptameric (PA^{7mer}) ring called the prepore. This prepore is able to bind up to three molecules of EF and/or LF, thus leading to a large hetero-oligomeric complex containing EF–LF–PA^{7mer} and receptors (for reviews see Abrami et al., 2005; Scobie and Young, 2005). Once formed, this complex is rapidly internalized via a pathway that depends both on lipid rafts and clathrin (Abrami et al., 2003). The complex is then delivered to early endosomes, where it associates with intraluminal vesicles (Abrami et al., 2004). At the low pH of endosomes, the prepore undergoes a conformational change that leads to its membrane insertion and pore formation. Interestingly, the pH sensitivity is determined by the receptor, and a lower pH is required when PA^{7mer} is bound to CMG2 when compared with TEM8 (Rainey et al., 2005; Wolfe et al., 2005). Low pH also triggers partial unfolding of EF and LF, which can translocate across the PA^{7mer} channel (Krantz et al., 2005). Because channel formation appears to occur preferentially in the intraluminal vesicles of the multivesicular endosomes, EF and LF end up in the lumen of these vesicles (Abrami et al., 2004). Final release of EF and LF from endosomes to the cytoplasm requires back fusion events between intraluminal vesicles and the limiting membrane at the level of late endosomes (Abrami et al., 2004).

Because EF and LF are unable to bind to cells or cross membranes on their own, binding to PA is an absolute prerequisite for internalization and intoxication. Therefore, PA receptors must remain at the cell surface until heptamerization and binding of the enzymatic subunits have taken place. In fact, PA83 is poorly internalized in comparison with PA63 (for reviews see

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Abbreviations used in this paper: ßMCD, β-methylcyclodextrin; CMG2, capillary morphogenesis gene 2; DRM, detergent-resistant membrane; EF, edema factor; Endo H, endoglycosidase H; LF, lethal factor; PA, protective antigen; TEM8, tumor endothelial marker 8; Ub, ubiquitin; WT, wild type.

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Collier and Young, 2003; Abrami et al., 2005; Scobie and Young, 2005) as a result of a differential localization on the plasma membrane (Abrami et al., 2003). Whereas PA83 localizes to the glycerophospholipidic region, PA63 (in particular the heptameric form) is found in specialized cholesterol-rich domains called lipid rafts. This raft association is essential for subsequent internalization of the toxin (Abrami et al., 2003). Therefore, although it is receptor mediated, anthrax toxin endocytosis is actually toxin driven.

To understand the molecular mechanisms that govern this well-orchestrated behavior of anthrax toxin receptors at the cell surface, we investigated the roles of posttranslational modifications of the receptor cytoplasmic tails. We focused on two modifications: S-palmitoylation and ubiquitination. S-palmitoylation is a reversible lipid modification involving the addition of a saturated 16-carbon palmitate moiety to specific cysteines via a thioester linkage. This modification is used by several hydrophilic proteins such as Ras to associate with membranes but is also found in transmembrane proteins such as the transferrin receptor (Alvarez et al., 1990), LAT (linker protein for activation of T cells; Zhang et al., 1998), influenza HA (Scheiffele et al., 1997), or the G protein of the vesicular stomatitis virus (Mack and Kruppa, 1988). The exact function of this modification in membrane proteins is mostly unclear, but it might modulate the interaction of these proteins with membranes or membrane domains (Smotrys and Linder, 2004) as well as with other proteins.

Ubiquitination is the addition of a ubiquitin (Ub) moiety to cytoplasmic lysines by E3 Ub ligases, the third enzymes in the ubiquitination pathway (Hicke and Dunn, 2003). This added Ub itself may or may not be subsequently ubiquitinated on Lys^{48} or on Lys^{63} leading to polyubiquitin chains (Haglund and Dikic, 2005). Although polyubiquitination on Lys^{48} is essentially involved in the degradation of proteins by the proteasome, monoubiquitination (the addition of a single Ub moiety to one or multiple lysines) as well as polyubiquitination on Lys^{63} have been shown to be involved in endocytosis of plasma membrane proteins (G-coupled receptors, growth factors, and transporters; Hicke and Dunn, 2003) and sorting of internalized receptors into multivesicular bodies (Katzmann et al., 2002).

We report that TEM8 and CMG2 are targets for both palmitoylation and ubiquitination and that these two modifications, via counteracting effects, control receptor endocytosis.

Results

Characterization of TEM8/1-HA and CMG2/4-V5

TEM8 and CMG2 both exist as four isoforms, two of which, in each case, act as anthrax toxin receptors: isoforms 1 and 2 of TEM8 (TEM8/1 and TEM8/2) and isoforms 1 and 4 of CMG2 (CMG2/1 and CMG2/4; for review see Scobie and Young, 2005). These isoforms differ only in their cytoplasmic tails (Fig. 1 A); TEM8/2 has a far shorter cytoplasmic tail than TEM8/1 and CMG2/1, and CMG2/4 differs only in the last 12–13 residues. Even between TEM8 and CMG2, the long tails have a very high degree of conservation (Fig. 1 A). Therefore, we have focused this study mainly on TEM8/1 but repeated key experiments with the short TEM8/2 isoform as well as with CMG2/4.

TEM8/1 was tagged with an HA epitope at the COOH terminus and was either transiently transfected or stably expressed in receptor-deficient CHO cells ($CHO^{\Delta ATR}$; Liu and Leppla, 2003). Expression levels were overall higher upon transient transfection (Fig. 1 B) but were still readily detectable in the stably TEM8/1-HA–expressing cells (Fig. 1, B and C). Most of the expressed receptor was present at the cell surface as indicated by its sensitivity to cell surface trypsinization (Fig. 1 D). TEM8-HA always migrated as a doublet with an upper smeared band (Fig. 1 B, m) and a lower well-defined band (Fig. 1 B, p), the intensity of which varied greatly from experiment to experiment. We investigated whether this migration pattern was caused by glycosylation because the extracellular domain of TEM8 has three predicted *N*-glycosylation sites. Treatment of cells with tunicamycin, an inhibitor of *N*-glycosylation, led to the appearance of a third, lower mobility band (u; unglycosilated) with a concomitant decrease in the intensity of bands m and p (Fig. 1 E). *N*-glycosidase F treatment of control cell extracts led to the complete disappearance of bands m and p to the benefit of band u (Fig. 1 E), showing that m and p both correspond to glycosylated TEM8. Band m was endoglycosidase H (Endo H) resistant, and band p was Endo H sensitive (Fig. 1 E). This shows that TEM8 in band m (mature) had acquired complex Golgi-modified sugars, whereas that in band p (precursor) contained incompletely modified sugars.

CMG2/4 was expressed with a COOH-terminal V5 tag (Dowling et al., 2003). As for TEM8, CMG2/4-V5 migrated as a smear plus a lower molecular weight band (Fig. 1 F). Both bands were sensitive to *N*-glycosidase F treatment, and, as for TEM8, only the lower band was sensitive to Endo H (Fig. 1 F). Altogether, this indicates that CMG2/4 is also glycosylated, as predicted by the presence of two *N*-glycosylation sites in the ectodomain.

PA DRM association is palmitoylation dependent

We have previously shown that the anthrax toxin modifies the surface distribution of its receptor, inducing its clustering in cholesterol-rich raftlike domains. We wondered whether posttranslational modifications of the receptor tail could be involved in regulating interactions of TEM8 and CMG2 with membrane domains.

TEM8/1 and /2 as well as CMG2/1 and /4 all contain cysteine residues in their cytoplasmic tails, which are potential sites for S-palmitoylation (Linder and Deschenes, 2004). More specifically, all four proteins have two conserved juxtamembranous cysteines (Fig. 1 A; Cys-346 and Cys-347 in TEM8), a third cysteine is conserved between TEM8/1 (Cys-481) and CMG2, and a fourth unconserved cysteine is found in TEM8/1 (Cys-521) and CMG2/1 (Cys482).

To test whether palmitoylation plays a role in anthrax toxin endocytosis, we analyzed whether the palmitoylation inhibitor 2-bromopalmitate (Webb et al., 2000) would affect the raft association of PA63 in BHK cells, a cell line that expresses transmembrane isoforms of both TEM8 and CMG2 (unpublished data).

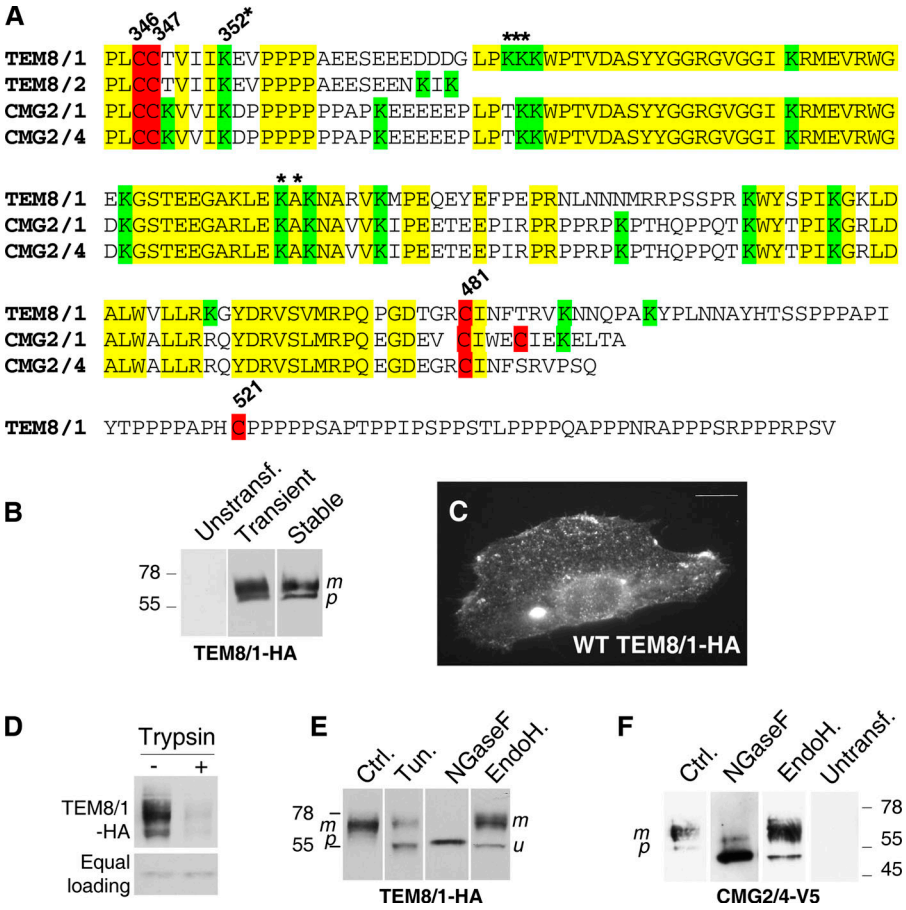

Figure 1. **TEM8/1 and CMG2/4 are glycosylated.** (A) Alignment of the cytoplasmic tails of human TEM8 and human CMG2 using the SIM software of the EXPASY server (www.expasy.ch). Regions of identity are shown in yellow, lysine residues in green, and cysteine residues in red. Numbering of the residues corresponds to that of TEM8/1. The asterisks label the lysine mutants in the K6R mutant described in Fig. 7. (B and C) CHO$^{\Delta ATR}$ cells untransfected and stably or transiently transfected (for 48 h; B) with a TEM8/1-HA construct were analyzed by Western blotting (40 μg of protein/lane; B) or immunofluorescence (C) using an anti-HA antibody. Bar, 10 μM. (D) CHO$^{\Delta ATR}$ cells stably expressing TEM8/1-HA were submitted or unsubmitted to surface trypsinization at 4°C and subsequently analyzed for the presence of TEM8/1-HA by Western blotting. (E) CHO$^{\Delta ATR}$ cells transiently expressing TEM8/1-HA were grown in the presence or absence of tunicamycin. Control cell extracts were subsequently left untreated or treated with *N*-glycosidase F or Endo H. The effects of these treatments were analyzed by SDS-PAGE followed by Western blotting against the HA tag. (B and E) Band u, unglycosilated form; p, glycosylated precursor; m, mature form. (F) Extracts of CHO$^{\Delta ATR}$ cells transiently expressing or not expressing CMG2/4-V5 were left untreated or treated with *N*-glycosidase F or Endo H and subsequently analyzed by SDS-PAGE followed by Western blotting against the V5 tag.

We first verified that PA binding was not affected by the treatment (Fig. 2 A). Raft association was then monitored by following the association with detergent-resistant membranes (DRMs; Brown and London, 1998). PA63 was associated with DRMs in control cells as previously observed (Abrami et al., 2003) but shifted to the detergent-soluble fractions in drug-treated cells (Fig. 2 B). This was not caused by a general disruption of lipid rafts because glycosyl-phosphatidylinositol–anchored proteins, followed here using the glycosyl-phosphatidylinositol–specific bacterial toxin aerolysin (Fivaz et al., 2002) as well as caveolin-1, remained primarily in the DRM fractions. Although caveolin-1 is palmitoylated on three cysteines, the modification is not required for DRM association (Dietzen et al., 1995). The inhibitory effect of 2-bromopalmitate on PA63-raft association was also accompanied by the inhibition of endocytosis as reflected by a lack of SDS-resistant PA^{7mer} pore and the absence of LF-mediated cleavage of one of the LF substrates, the MAPK kinase MEK1 (Fig. 2 C; for review see Collier and Young, 2003).

To test whether 2-bromopalmitate inhibited the heptamerization process itself, we designed an assay to detect cell surface–formed prepores. These are SDS sensitive and, therefore, are not detected by SDS-PAGE. However, they can be converted to the SDS-resistant phenotype by submitting cell extracts to low pH (pH 4.5) before SDS-PAGE analysis, as illustrated in Fig. 2 C (middle left) for control cells. The prepore was undetectable for 2-bromopalmitate–treated cells (Fig. 2 C).

TEM8 and CMG2 are palmitoylated

The aforementioned experiments show that palmitoylation events are important for anthrax toxin raft association and heptamerization. To determine whether the receptors themselves are palmitoylated, TEM8/1-HA and CMG2/4-V5 were immunoprecipitated from lysates of ^{3}H-palmitic acid–labeled CHO$^{\Delta ATR}$ cells transiently transfected with the respective constructs. Radiolabeled bands with motilities similar to that of TEM8/1-HA (Fig. 3 A) and CMG2/4-V5 (Fig. 3 B), respectively, were detected. This band was sensitive to in vitro hydroxylamine treatment

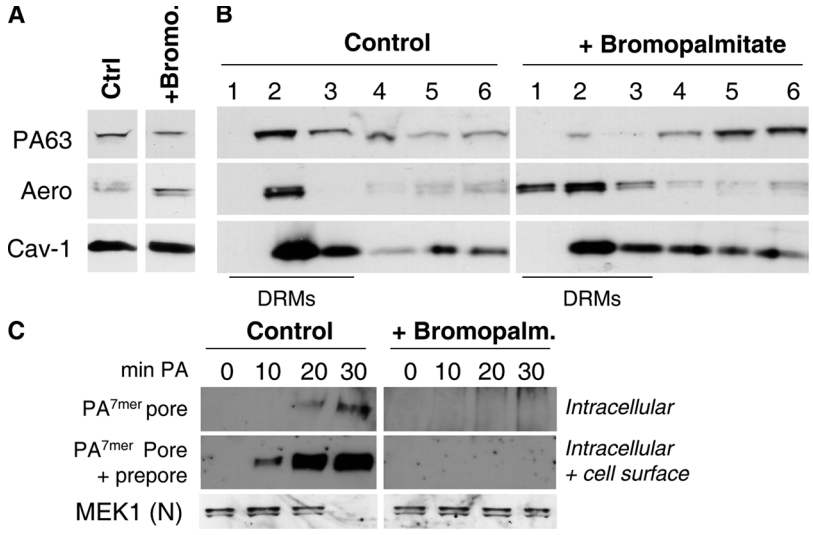

Figure 2. **Palmitoylation events are required for DRM association and internalization of PA.** (A and B) Control BHK cells were pretreated or untreated with bromopalmitate and were incubated with 500 ng/ml nicked PA63 and 20 ng/ml aerolysin for 1 h at 4°C followed by 10 min at 37°C. (A) Cell extracts were submitted to SDS-PAGE followed by Western blotting to reveal PA63, aerolysin, and caveolin-1 (Cav-1). (B) Cells were solubilized in 1% Triton X-100, run on an OptiPrep gradient, and each fraction was analyzed by SDS-PAGE followed by Western blotting against PA, aerolysin, and caveolin-1. (C) BHK cells were pretreated with bromopalmitate and incubated with 500 ng/ml nicked PA63 for 1 h at 4°C followed by different times at 37°C. Cell extracts (40 μg of protein) were analyzed by SDS-PAGE and Western blotting to reveal the SDS-resistant PA^{7mer} pore and MEK1 (NH_2-terminal directed). To detect the prepore (SDS-sensitive nonmembrane-inserted PA^{7mer}), cell extracts were submitted to an acid pulse before SDS analysis.

(Fig. 3 A) or cellular treatment with 2-bromopalmitate (Fig. 3 C) as shown for TEM8/1-HA, indicating that palmitate addition occurred via a thioester bond. The short isoform of TEM8 containing only two cysteines was also palmitoylated (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200507067/DC1), as was CMG2/1 (not depicted).

To investigate when during the life cycle of TEM8/1 palmitoylation occurred, ^{3}H-palmitate labeling was performed either in the presence of cycloheximide to inhibit protein synthesis or in the presence of brefeldin A to inhibit transport from the endoplasmic reticulum to the Golgi. Neither drug significantly affected the amount of immunoprecipitated receptor (Fig. 3 C, anti-HA Western blot). However, both drugs led to an ~50% reduction in ^{3}H-palmitate incorporation, as quantified by densitometry, but not to full inhibition (Fig. 3, C and D), suggesting that palmitoylation occurs both in the early secretory pathway and later in the life cycle of the protein. A pulse-chase performed after ^{3}H-palmitate incorporation showed that the palmitate groups were lost within an hour (Fig. 3 E). This loss was caused by the reversible nature of the modification and not by degradation of the protein because a pulse-chase experiment using [^{35}S]cysteine/methionine showed that the half-life of TEM8/1-HA in these experiments well exceeded 5 h (Fig. 3 E; same ^{35}S curve as in Fig. 5 B).

TEM8/1 is palmitoylated on multiple cysteines

To investigate which of the four TEM8/1 cytoplasmic cysteines can be palmitoylated, we first concentrated on the two juxtamembrane cysteines because TEM8/2, which contains only these two cysteines, is palmitoylated (Fig. S1). Moreover, palmitoylation sites adjacent to the transmembrane region have been

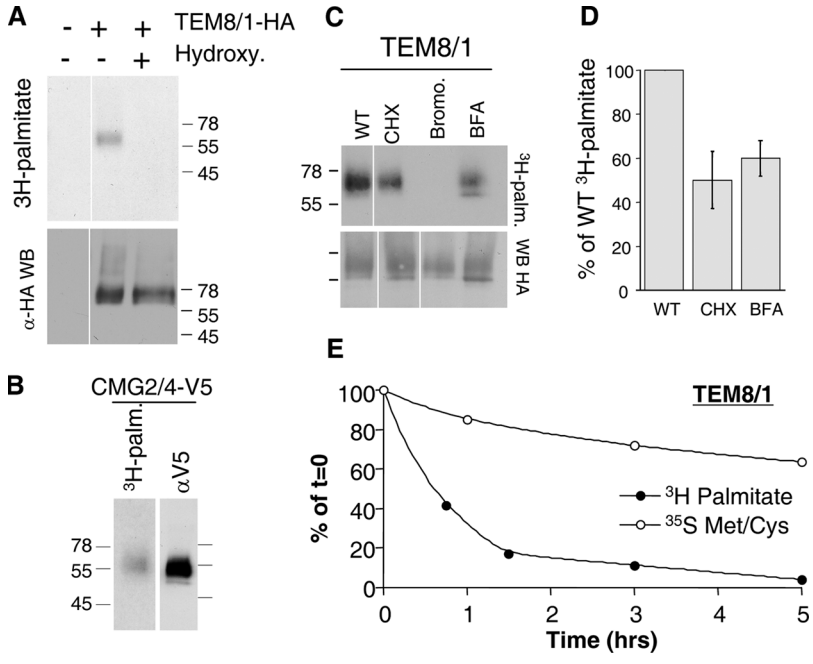

Figure 3. **TEM8/1 and CMG2/4 anthrax toxin receptors are palmitoylated.** $CHO^{\Delta ATR}$ cells transfected with TEM8/1-HA (A) or CMG2/4-V5 (B) were incubated with ^{3}H-palmitic acid for 2 h before immunoprecipitation using anti-tag antibodies. Immunoprecipitates were split into two, run on 4–20% gels, and analyzed either by autoradiography (^{3}H-palmitate) or Western blotting (anti-tag HA or V5). (C) Before ^{3}H-palmitic acid incorporation, TEM8/1-HA–expressing cells were pretreated either with cycloheximide, 2-bromopalmitate, or brefeldin A and were analyzed by autoradiography (^{3}H-palmitate) or Western blotting (anti-tag HA). (D) Autoradiograms from C were quantified by densitometry using ScanAnalysis software (Biosoft). Error bars correspond to SD (n = 3). (E) $CHO^{\Delta ATR}$ cells transiently transfected for 48 h with TEM8/1-HA cDNA were pulsed either for 2 h with ^{3}H-palmitic acid or for 30 min with [^{35}S]cysteine/methionine and were chased for different times. After anti-HA immunoprecipitation, samples were analyzed by SDS-PAGE followed by autoradiography and densitometry (^{3}H) or Phosphoimager analysis (^{35}S; same curve as in Fig. 5 B). Results were normalized to the values at time = 0.

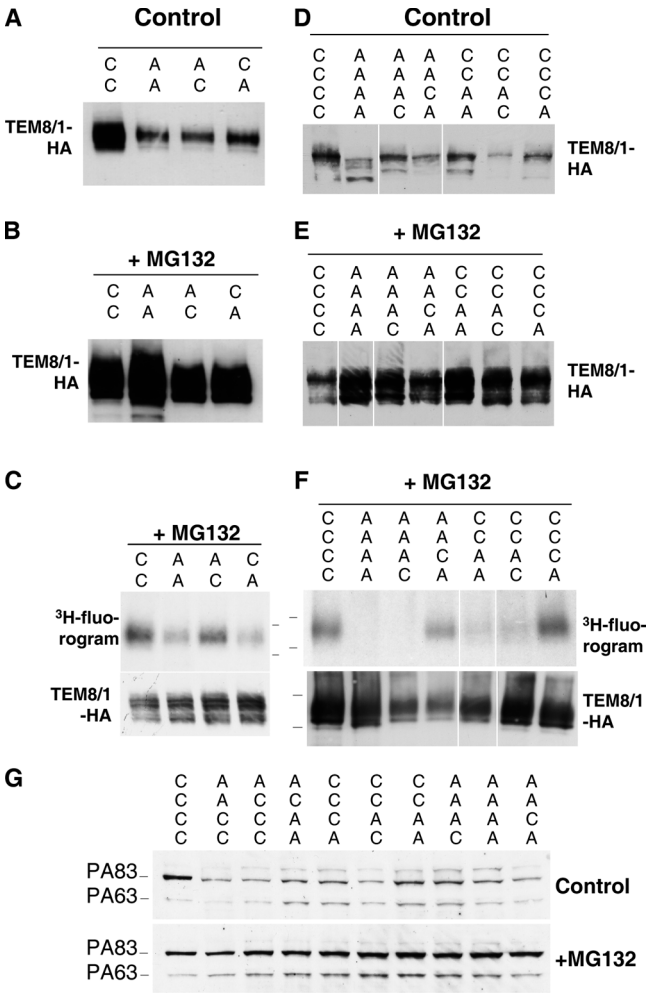

Figure 4. **TEM8/1 is palmitoylated on multiple cysteines.** (A–C) CHO$^{\Delta ATR}$ cells were transiently transfected for 48 h with WT (CC) or mutant TEM8/1-HA cDNA in which one or both of the first two cytoplasmic cysteines were changed to alanine (mutants AC, CA, and AA). Cells were treated (B) or untreated (A) with the proteasome inhibitor MG132. 40 µg of cell extracts were analyzed by SDS-PAGE and Western blotting against the HA tag. (C) CHO$^{\Delta ATR}$ cells transfected with WT or mutant TEM8/1-HA were incubated with MG132 and ^{3}H-palmitic acid for 2 h and submitted to immunoprecipitation against the HA tag. Samples were analyzed by SDS-PAGE followed by autoradiography and Western blotting against HA. (D–F) Similar experiments were performed on single to quadruple mutants of the four cysteine residues. (G) CHO$^{\Delta ATR}$ cells transiently transfected for 48 h with WT (CCCC) or mutant TEM8/1-HA cDNAs were incubated with 500 ng/ml PA83 for 1 h at 4°C followed by 30 min at 37°C. 40 µg of cell extracts were analyzed by SDS-PAGE and Western blotting against PA.

previously reported for several proteins such as CD4 (Bijlmakers and Marsh, 2003) or members of the SNARE family of membrane fusion proteins (Rothman, 1994). Cysteines at positions 346 and 347 in TEM8/1 were changed to alanine in single (mutants AC and CA) and double (mutant AA) mutants. All three mutants were expressed to lower levels than wild type (WT; Fig. 4 A). This was not a result of lower transfection efficiencies because equivalent TEM8/1 expression levels were observed when treating cells with the proteasome inhibitor MG132 (Fig. 4 B).

To compare cells that express similar amounts of receptor and have expression levels that allow the detection of ^{3}H-palmitate, incorporation was performed on MG132-treated cells. Only when the second cysteine was modified were lowerlevels of ^{3}H-palmitate incorporation observed (Fig. 4 C), indicating that Cys-347is palmitoylated in the WT protein. The observation thatthe double mutant still incorporated significant amounts of ^{3}H-palmitate, however, indicated that other cysteines were modified. Thus, Cys-481 and Cys-521 in TEM8/1 were also changed to alanine in single to quadruple mutants. Once more, all mutants were expressed at lower levels than the WT receptor(Fig. 4 D), an effect that could similarly be overcome by treating cells with MG132 (Fig. 4 E). These mutants were expressed at the cell surface as indicated by their ability to bind PA (Fig. 4 G).

The quadruple mutant AAAA and the AAAC mutant did not incorporate ^{3}H-palmitate (Fig. 4 F). However, the fact that the CCCA mutant in repeated experiments had a somewhat lower incorporation than the WT TEM8/1 suggests that Cys-521 can be palmitoylated, possibly in a subpopulation of receptors, but only when other palmitoylation sites are present. The AAC^{481}A mutant was always significantly modified, as were all of the mutants with a cysteine at position 481. Thus, repeated palmitoylation experiments showed that mutations of Cys-347 and Cys-481 always led to a drastic decrease in ^{3}H-palmitate incorporation, whereas the mutation of Cys-521 had a milder but significant effect. It has previously been observed that the mutation of palmitoylation sites leads to the aberrant palmitoylation of remaining cysteines, especially in double-cysteine motifs (Percherancier et al., 2001; Sims and Wiedmer, 2001; Wiedmer et al., 2003). Therefore, we focused further experiments on the quadruple AAAA mutant in comparison with the WT (CCCC) receptor.

Palmitoylation modulates the half-life of TEM8

The lower expression levels of all palmitoylation mutants when compared with WT suggested that palmitoylation affects the half-life of the receptor. Therefore, pulse-chase experiments using [^{35}S]cysteine/methionine labeling were performed in transiently transfected CHO$^{\Delta ATR}$ cells. The initial level of synthesis was very similar for the WT and AAAA mutant receptors (Fig. 5 A). However, 50% of the AAAA mutant was lost after ~130 min (Fig. 5 B), whereas >60% of the WT receptors were still present after 5 h. The 30% loss in AAAA TEM8/1 during the first hour of chase (Fig. 5, A and B) suggests that palmitoylation might somewhat affect folding/trafficking through the early secretory pathway. Because this cannot account for the drastic reduction in receptor half-life, we investigated whether defective palmitoylation could cause premature targeting of TEM8/1 to lysosomes.

We first generated stable cell lines expressing AAAA TEM8/1 and found, as expected, lower steady-state expression levels (Fig. 5 C). Cells were then fed with an inhibitor of lysosomal enzymes, leupeptin (inhibitor of serine and cysteine proteases). This treatment led to some protection of the WT receptor (at the low exposure shown in Fig. 5 D, CCCC TEM8/1 was only detected in leupeptin-treated cells). However, the effect was far more pronounced for AAAA TEM8/1 (Fig. 5 D). Intracellular accumulation was confirmed by immunofluorescence

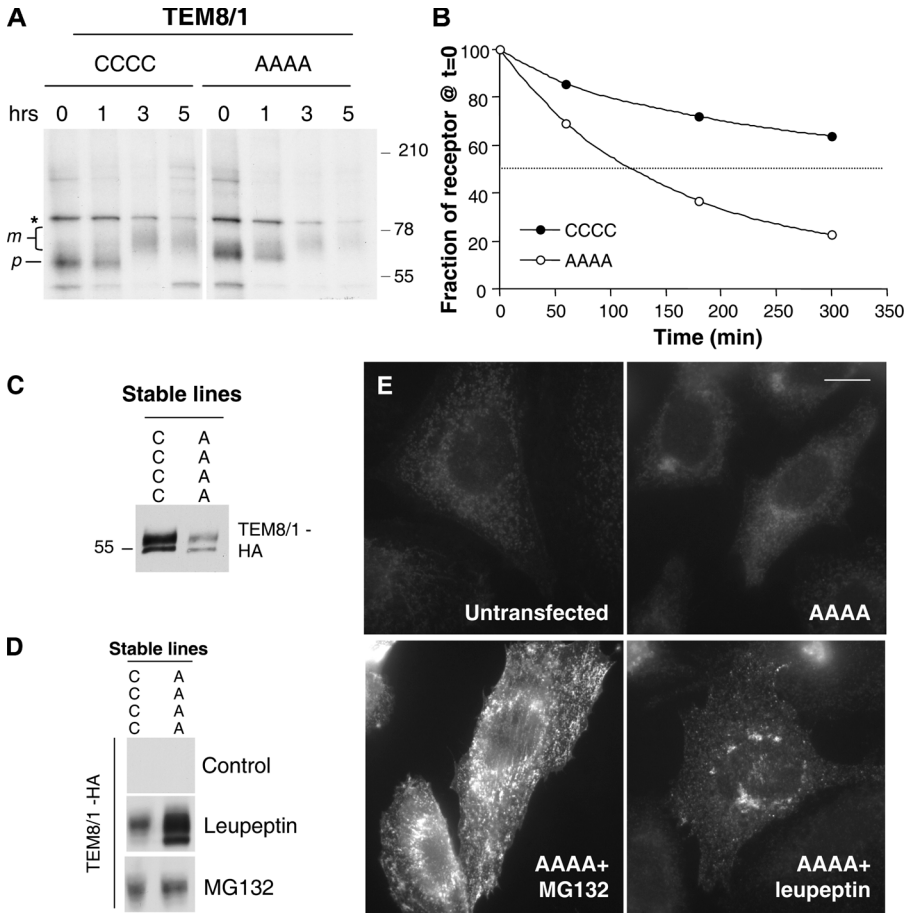

Figure 5. **Palmitoylation-deficient TEM8/1 has a reduced half-life.** (A) CHO$^{\Delta ATR}$ cells transiently transfected for 30 h with plasmids expressing WT or AAAA TEM8/1 were submitted to a pulse-chase analysis with [^{35}S]methionine/cysteine. Immunoprecipitated receptors were followed by autoradiography (8-d exposure). Mature TEM8 is labeled m, and the Endo H–sensitive precursor is labeled p. The band labeled with an asterisk is an unknown coimmunoprecipitated protein that is not detected in other cells, such as HeLa. (B) TEM8/1 radioactivity was quantified using a Phosphoimager (Bio-Rad Laboratories). Results correspond to the mean of two experiments and were normalized to the radioactivity at time = 0. (C–E) Cell extract for CHO$^{\Delta ATR}$ cells stably expressing WT (CCCC) or mutant AAAA TEM8/1-HA were analyzed by Western blotting (C and D) or immunofluorescence (E) against HA. (D and E) Cells were left untreated, treated with MG132, or fed with leupeptin. In E, the exposure times were identical for all images, but a 75% cut-off filter on the excitation beam was used for the MG132 condition. Untransfected CHO$^{\Delta ATR}$ cells are shown for comparison. Bar, 10 μM.

(Fig. 5 E). Although in the absence of treatment, AAAA TEM8/1-HA was undetectable by fluorescence microscopy, leupeptin feeding led to the appearance of punctate perinuclear structures (Fig. 5 E), which were presumably late endosomes/lysosomes.

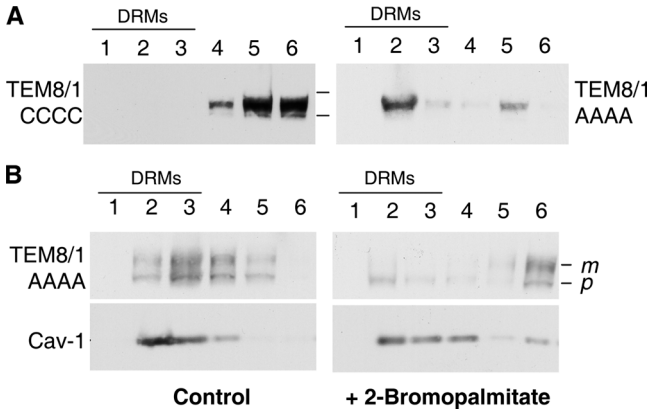

Figure 6. **Palmitoylation-deficient TEM8/1 associates constitutively with DRMs.** (A) DRMs were prepared from CHO$^{\Delta ATR}$ cells stably expressing CCCC or AAAA TEM8/1-HA, and the distribution of receptors was analyzed by Western blotting against HA. A higher exposure is shown for the AAAA mutant. (B) CHO$^{\Delta ATR}$ cells stably expressing AAAA TEM8/1-HA were left untreated or treated with 2-bromopalmitate. DRMs were prepared, and the distribution of mutant TEM8/1-HA and caveolin-1 was analyzed by Western blotting. Mature TEM8/1-HA is labeled m, and the Endo H–sensitive precursor is labeled p.

This was in contrast with the effect of MG132, which also led to a massive increase in staining but primarily at the plasma membrane (Fig. 5 E). Thus, palmitoylation of TEM8/1 appears to be crucial in preventing premature targeting to lysosomes.

Palmitoylation is a negative regulator of TEM8 DRM association

We next investigated whether the palmitoylation of TEM8/1 is involved in regulating its association with DRMs (Abrami et al., 2003). Stably expressed WT TEM8/1 was found in detergent-sensitive fractions (Fig. 6 A, left) as previously described (Abrami et al., 2003). In contrast, AAAA TEM8/1 was almost entirely associated with DRMs (Fig. 6 A, right). These observations indicate that palmitoylation acts as a negative regulator of TEM8/1 DRM association. The drastic difference between the AAAA mutant and the WT receptor suggests that the bulk of the WT receptor is palmitoylated at steady state. These observations also raise the interesting possibility, which is not addressed in this study, that depalmitoylation by a protein-thioesterase activity could be a mechanism for the regulation of raft association. Regulation of protein localization by palmitoylation/depalmitoylation has been proposed for soluble proteins in particular (Drenan et al., 2005; Rocks et al., 2005).

The observation that palmitoylation-deficient TEM8/1 (AAAA) is exclusively found in DRMs (Fig. 6 A) is in apparent contradiction with the fact that 2-bromopalmitate inhibited

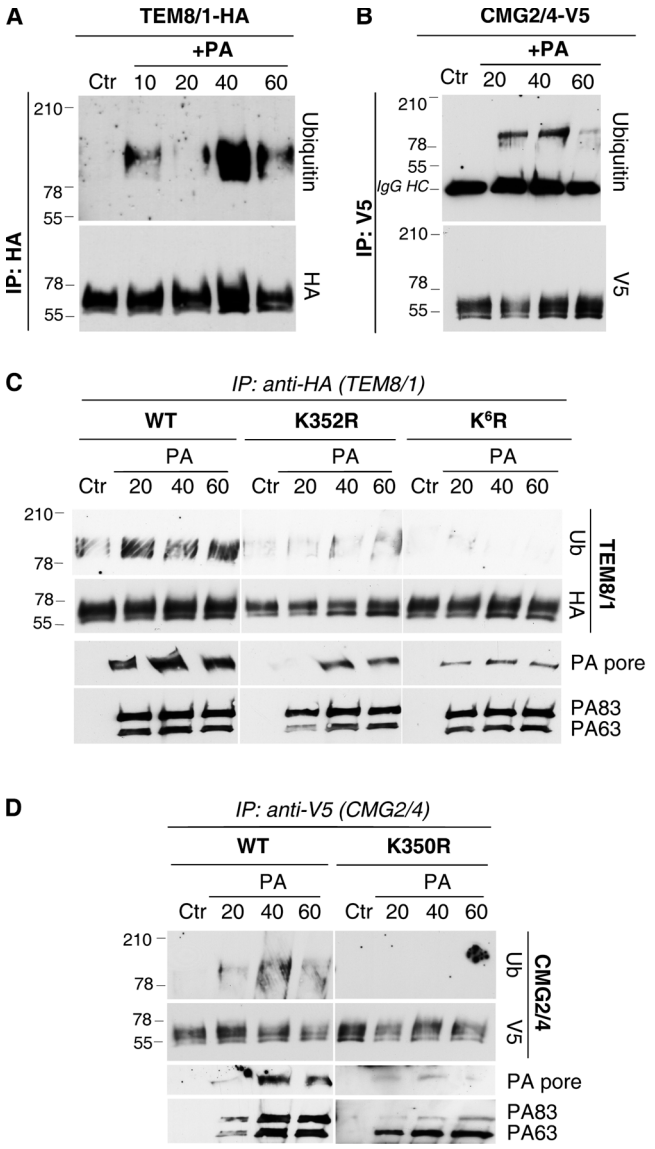

Figure 7. **PA triggers ubiquitination of its receptor.** CHO$^{\Delta ATR}$ cells were transiently transfected for 48 h with WT (A and B) or mutant forms (C and D) of TEM8/1-HA or CMG2/4-V5. 1 μg/ml PA83 was either added or not added to cells for 1 h at 4°C and shifted for different times to 37°C. After immunoprecipitation against HA (A and C) or V5 (B and D), samples were analyzed by Western blotting using anti-Ub, anti-HA, or V5 and anti-PA. In TEM8/1 K6R, lysines 352, 372, 373, 374, 412, and 414 were changed to arginine. HC, heavy chain.

DRM association of PA63 (Fig. 2 B). Therefore, we tested whether the drug would also affect the DRM association of AAAA TEM8/1. The mature Endo H–resistant form of the mutant receptor entirely relocalized to the detergent-soluble fractions (Fig. 6 B), indicating the involvement of a palmitoylation event in the association of AAAA TEM8/1 with DRMs, the substrate of which must be a protein other than the receptor itself.

PA induced ubiquitination of anthrax toxin receptors

We have previously shown that heptamerization of PA not only triggers the redistribution of the toxin–receptor complex to lipid rafts but also triggers its rapid endocytosis (Abrami et al., 2003). We investigated whether this toxin-induced uptake could be caused by a second posttranslational modification of the receptor cytoplasmic tail that would be raft dependent. We focused our attention on ubiquitination (Haglund and Dikic, 2005). CHO$^{\Delta ATR}$ cells transiently transfected with TEM8/1-HA or CMG2/4-V5 were treated with PA for various times. After immunoprecipitation of the receptors, Western blots were performed with anti-tag and anti-Ub antibodies. The addition of the toxin clearly led to the appearance of a smeared Ub-positive band both for TEM8/1 (Fig. 7 A) and for CMG2/4 (Fig. 7 B). The absence of ladder was suggestive of monoubiquitination rather than polyubiquitination with long chains, as observed for Ub^{48} ubiquitination and proteasomal degradation (Haglund and Dikic, 2005). Experiments performed with the shorter TEM8/2 isoform similarly led to the detection of a ubiquitinated band (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200507067/DC1). The ubiquitinated band detected for TEM8/2 was smaller than that detected for TEM8/1, suggesting that the receptor itself was the modified protein rather than an interacting partner.

To confirm this, lysine mutagenesis was performed. Of the 16 lysines in TEM8/1 and 14 in CMG2/4, we first changed Lys-352 to arginine in TEM8 and the corresponding Lys-350 in CMG2/4 because (1) it is the only lysine common to TEM8/1 and TEM8/2; (2) it is conserved between TEM8 and CMG2; and (3) Valdez-Taubas and Pelham (2005) suggested that the palmitoylation of Tlg1 prevents access of juxtamembranous lysines by E3 ligases. Although the ubiquitinated band could still be detected after the addition of PA to K352R TEM8/1 expression cells, ubiquitination was greatly diminished (Fig. 7 C). A stronger effect was obtained when mutating 6 of the 16 lysines to arginine (K^{6}R mutant in which all lysines labeled with an asterisk in Fig. 1 A were changed to arginine, including Lys-352). These experiments confirm that TEM8/1 is the substrate of the ubiquitination reaction and that Lys-352 is one of the modified sites but that additional lysines might be modified. The effect of mutating the first conserved juxtamembranous lysine to arginine was even more drastic in CMG2/4. No Ub-positive band could be detected in PA-treated K350R CMG2/4–transfected cells (Fig. 7 D).

To investigate whether ubiquitination of the receptor is important for endocytosis of the anthrax toxin, we monitored the appearance of the SDS-resistant PA^{7mer} pore in lysine mutant-expressing cells. As shown in Fig. 7 (C and D), the appearance of the SDS-resistant pore was either delayed or strongly diminished in the TEM8/1 and CMG2/4 lysine mutant–expressing cells, demonstrating that ubiquitination of the receptor is important for efficient endocytosis.

TEM8 ubiquitination is DRM mediated

Because endocytosis of TEM8 requires both raft association (Abrami et al., 2003) and ubiquitination (Fig. 7), we investigated whether these two events were linked. DRMs were isolated from transiently transfected CHO$^{\Delta ATR}$ cells. The bulk of TEM8/1 was found in detergent-soluble fractions (Fig. 8 A, left), as also observed in Fig. 6 A. In marked contrast, ubiquinated TEM8/1 was detected exclusively in DRMs (Fig. 8 A, left).

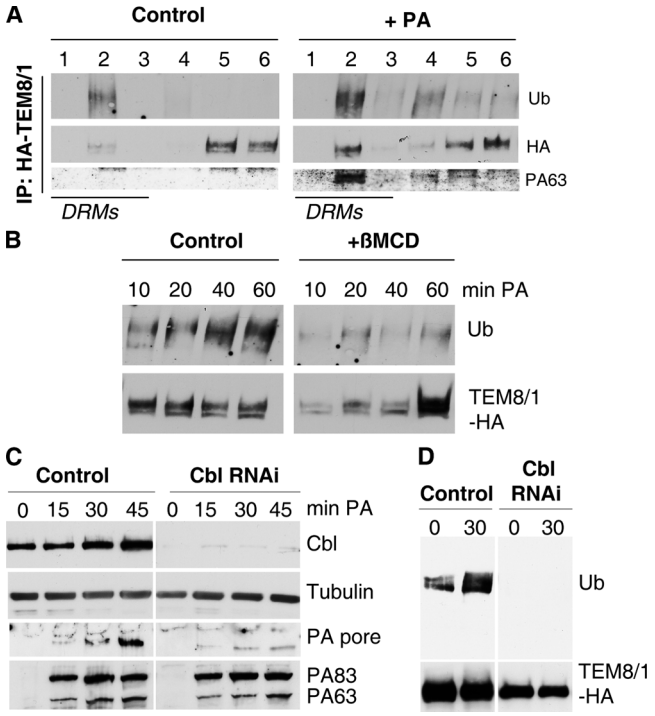

Figure 8. **Endocytosis of anthrax toxin receptor requires DRM-mediated ubiquitination and the E3 ligase Cbl.** (A) CHO$^{\Delta ATR}$ cells transfected for 48 h with WT TEM8/1-HA were incubated with 1 μg/ml PA83 for 1 h at 4°C followed by 40 min at 37°C, solubilized in Triton X-100 at 4°C, and separated on an OptiPrep gradient. TEM8/1-HA was immunoprecipitated from each fraction and analyzed by SDS-PAGE and Western blotting using anti-Ub, anti-HA, and anti-PA antibodies. (B) CHO$^{\Delta ATR}$ cells transfected for 48 h with WT TEM8/1-HA were treated with βMCD to extract cholesterol, incubated with 1 μg/ml PA83 for 1 h at 4°C, and shifted for different times at 37°C. After immunoprecipitation with anti-HA beads, samples were analyzed by Western blotting using anti-Ub and anti-HA antibodies. (C) HeLa cells were transfected or untransfected with siRNAs against Cbl for 72 h and incubated with 500 ng/ml PA83 for different times at 37°C. Cell extracts were blotted for Cbl, tubulin (as an equal loading marker), and PA. (D) HeLa cells were untransfected or transfected with siRNAs against Cbl for a total of 72 h in total. 24 h later, these cells were additionally transfected with TEM8/1-HA for 48 h and incubated with 500 ng/ml PA83 for different times at 37°C. TEM8/1-HA was immunoprecipitated from each fraction and analyzed by SDS-PAGE and Western blotting using anti-Ub and anti-HA antibodies.

When cells were treated with the toxin before Triton X-100 solubilization, PA63 was associated with DRMs (as in Fig. 2 B) and led to the recruitment of toxin-bound TEM8/1 to this fraction (Fig. 8 A, right). Concomitantly, the ubiquitinated form of TEM8/1 was increased in the same fraction (Fig. 8 A). Raft impairment by cholesterol extraction using the sequestering agent β-methylcyclodextrin (ßMCD) led to a strong inhibition of PA-induced TEM8/1 ubiquitination (Fig. 8 B). This observation suggests that microdomain association precedes and is required for this posttranslational modification.

The E3 Ub ligase Cbl is required for anthrax toxin endocytosis

Cbl is an E3 Ub ligase that can interact with lipid rafts (Lafont and Simons, 2001; Haglund et al., 2004). To test for the involvement of Cbl in anthrax toxin endocytosis, we decided to perform RNA silencing. HeLa cells were used because of their human origin (the sequence of hamster Cbl is not available) and their high transfection efficiencies. These cells express TEM8 as indicated by the pH sensitivity of PA channel formation (Rainey et al., 2005). As shown in Fig. 8 C, Cbl could be efficiently silenced by this method. The absence of Cbl did not affect binding of the toxin as indicated by the unaltered presence of PA83/PA63. However, appearance of the PA^{7mer} pore was drastically inhibited (Fig. 8 C). To investigate the effect of Cbl RNA interference on the ubiquitination of TEM8 itself, RNA interference–treated cells were transfected with TEM8/1-HA, and toxin-induced ubiquitination after immunoprecipitation of the receptors was measured. As shown Fig. 8 D, the ubiquitinated form of TEM8/1 could not longer be detected. Theses experiments show that Cbl is responsible for the ubiquitination of TEM8/1 and its subsequent internalization.

Constitutive ubiquitination of palmitoylation-deficient TEM8/1 and the consequences as an anthrax toxin receptor

We found that AAAA TEM8/1 is constitutively associated with DRMs and that TEM8 ubiquitination is a raft-dependent modification. Therefore, we wondered whether AAAA TEM8/1 would be constitutively ubiquitinated. As shown in Fig. 9 A, the steady-state ubiquitination level of AAAA TEM8/1 was markedly higher than that of WT CCCC TEM8/1 (especially when comparing the levels of Ub vs. HA) both in stable cell lines and upon transient transfection (note that after immunoprecipitation of TEM8/1-HA from transiently transfected cells, the levels of expressed receptors seem to be similar even though analysis of total extracts shows a lower abundance of the AAAA mutant). Ubiquitination of AAAA TEM8/1 was dependent on the integrity of lipid rafts because the removal of cholesterol using ßMCD led to a drastic reduction in the level of AAAA TEM8/1 ubiquitination. This later observation also indicates that increased ubiquitination of AAAA TEM8/1 is not a consequence of misfolding of the cytoplasmic tail as a consequence of mutagenesis, because such an event would have been insensitive to acute cholesterol extraction from the plasma membrane.

Constitutive ubiquitination of the AAAA TEM8/1 and, thus, its constitutive endocytosis are likely to affect its ability to act as an anthrax toxin receptor. To address this issue directly, we monitored the cleavage kinetics of the LF target MEK1. Whereas MEK1 underwent LF-dependent cleavage in WT TEM8/1–expressing cells, the MAPK kinase remained intact in the AAAA TEM8/1–expressing cells during the time course of the experiment (Fig. 9 C). To test whether this lack of cleavage was only a result of the reduced number of surface-expressed receptors (levels of TEM-HA), we treated AAAA TEM8/1–expressing cells with a higher concentration of PA to reach similar amounts of bound PA as on WT TEM8/1–expressing cells (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200507067/DC1). Interestingly, even under these conditions, MEK1 cleavage in AAAA TEM8/1–expressing cells was minimal (Fig. S3), indicating that reduced PA binding does fully account for the reduced MEK1 cleavage in these cells.

Because AAAA TEM8/1 undergoes significant constitutive endocytosis, we tested whether it would mediate the

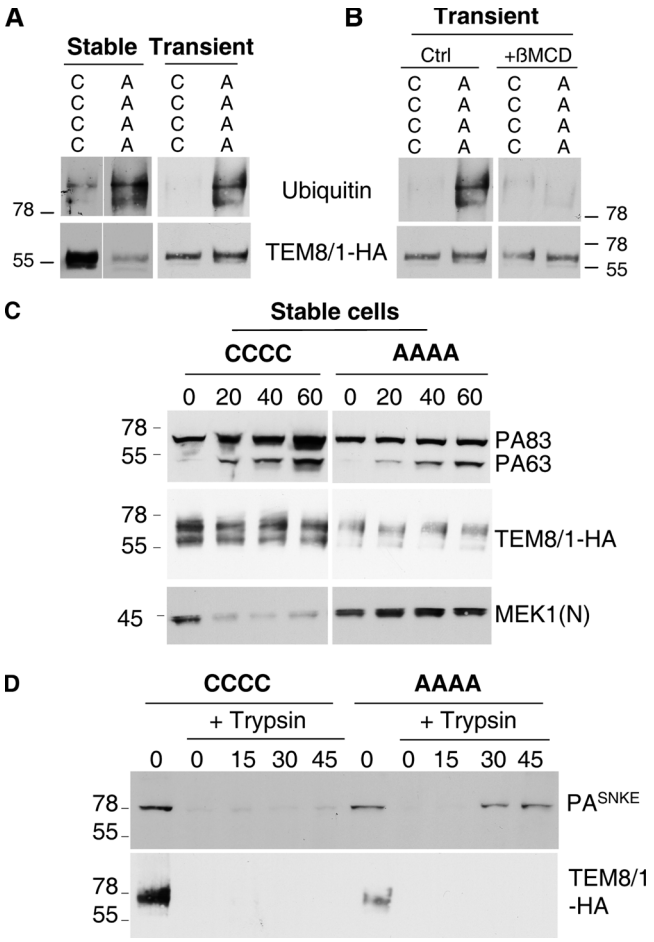

Figure 9. **Cells expressing the palmitoylation-deficient TEM8/1 are less sensitive to anthrax toxin.** (A and B) Anti-HA immunoprecipitation experiments were performed on $CHO^{\Delta ATR}$ cells stably or transiently (48 h) expressing CCCC or AAAA mutant TEM8/1-HA. Western blotting was performed against Ub and HA. (B) Cells were left untreated or treated with βMCD before lysis and immunoprecipitation. (C) $CHO^{\Delta ATR}$ cells stably expressing WT or AAAA TEM8/1-HA were treated with 500 ng/ml PA83 and 200 ng/ml LF at 37°C for different times. 40 μg of cell extracts were analyzed by SDS-PAGE followed by Western blotting against PA, HA, and MEK1 (NH_2-terminal–directed antibody). (D) $CHO^{\Delta ATR}$ cells transiently (48 h) expressing WT and AAAA mutant TEM8/1-HA were treated with 500 ng/ml of the furin-resistant PA^{SNKE} mutant for 1 h at 4°C and shifted to 37°C for different times. Surface-bound toxin was shaved off with trypsin (10 min at 37°Δ). To prevent lysosomal degradation of the internalized PA, cells were treated with 10 μM nocodazole to block microtubule-dependent transport to late endosomes. 40 μg of cell extracts were analyzed by SDS-PAGE and Western blotting against PA and HA.

internalization of PA83, which is an event that does not occur with the WT receptor and for which PA heptamerization is required (Abrami et al., 2003; Liu and Leppla, 2003). In this study, we made use of a mutant PA (PA^{SNKE}; Abrami et al., 2003) that is modified in the furin consensus cleavage site and, thus, remains in the PA83 form. As expected (Abrami et al., 2003), PA^{SNKE} was not internalized by WT receptors and was sensitive to surface trypsinization (Fig. 9 D). In contrast, all cell-bound PA^{SNKE} became trypsin resistant in AAAA TEM8/1–expressing cells, indicating that it had been completely endocytosed (Fig. 9 C, right).

Discussion

TEM8 and CMG2 are ideal anthrax toxin receptors because of their low steady-state endocytosis rate and rapid uptake upon clustering (Abrami et al., 2003). These properties guarantee that PA is not internalized unless it has heptamerized and, thus, bound EF and/or LF. In this study, we have analyzed the mechanisms that govern the cell surface behavior of the receptors.

Receptor palmitoylation

Although the number of cytoplasmic cysteines varies from two to four in the various transmembrane TEM8 and CMG2 isoforms, this study indicates that these isoforms are all palmitoylated at least on the second juxtamembranous cysteine. In addition, long isoforms, such as those shown here for TEM8/1, can be palmitoylated on the more distal cysteines. These different palmitoylation sites could be the substrate of more than one palmitoyl transferases, 23 of which have been identified in the human genome (Fukata et al., 2004; Linder and Deschenes, 2004). The first palmitoylation event appears to occur in the early secretory pathway, as indicated by the ∼50% inhibition of palmitate incorporation induced by cycloheximide or brefeldin A treatment and also indirectly suggested by the increased degradation of the palmitoylation-deficient mutant at early times after synthesis.

Based on the study of the palmitoylation-deficient AAAA TEM8/1 mutant, palmitoylation regulates the lifetime of TEM8/1 by preventing premature internalization and targeting to lysosomes. Lysosomal targeting in the absence of proper palmitoylation has previously been observed for other transmembrane proteins such as CCR5 (Percherancier et al., 2001) or the yeast SNARE Tlg1 (Valdez-Taubas and Pelham, 2005). Increased turnover of membrane proteins in the absence of normal palmitoylation, however, is not systematic because the yeast SNAREs Suc1 and Syn8 are not prematurely targeted to degradation in palmitoylation-deficient cells (Valdez-Taubas and Pelham, 2005).

However, the most surprising finding concerning the palmitoylation-deficient mutant was its high affinity for DRMs. This appears to be in contradiction with the accepted consensus in the lipid raft field that palmitoylation allows association with cholesterol-rich domains. Careful analysis of the data illustrates that the effect of palmitoylation on a transmembrane protein cannot be predicted because a variety of situations have been described. LAT is palmitoylated, and this modification is essential for DRM association (Zhang et al., 1998); caveolin-1 is triple palmitoylated, but this modification is not necessary for DRM association (Dietzen et al., 1995); and, finally, the transferrin receptor (Alvarez et al., 1990) and the G protein of the vesicular stomatitis virus (Mack and Kruppa, 1988), which are two well-characterized nonraft proteins, are both palmitoylated. We now illustrate a fourth scenario in which palmitoylation actually prevents DRM association.

Interesti5ngly, the DRM association of TEM8 was still sensitive to 2-bromopalmitate, indicating the requirement for other palmitoylated proteins. Based on this observation, we would like to propose that palmitoylation of TEM8 regulates its interaction with a partner protein, the palmitoylation of which is

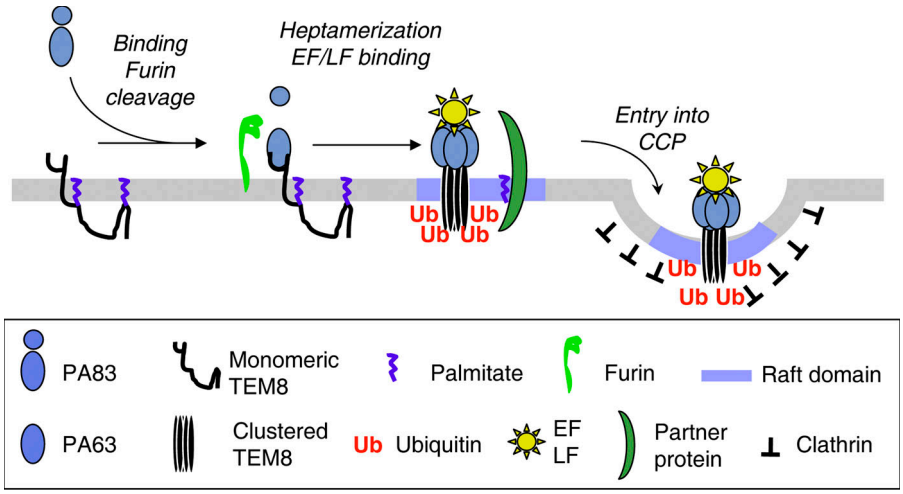

Figure 10. **Schematic representation of the toxin-induced behavior of TEM8/CMG2 at the cell surface.** Cell surface anthrax toxin receptor is palmitoylated and localizes to the glycerophospholipidic region of the membrane, where PA83 binds to it. Furin cleavage and heptamerization of PA trigger raft association of the toxin–receptor complex (Abrami et al., 2003) in a manner that probably involves a conformational change of the clustered receptors and the association with partner proteins, one of which is predicted to be palmitoylated. Within rafts, the receptors become accessible to its E3 Ub ligase, possibly Cbl that modifies the cytoplasmic tails, thus allowing the interaction with proteins of the endocytic machinery harboring Ub-interacting domains. The clathrin-dependent endocytic machinery is thus recruited, and the toxin–receptor complex is internalized.

required for the toxin-induced raft association of WT TEM8. This interaction would be essential for the toxin's action based on the inhibitory effect of 2-bromopalmitate on PA surface heptamerization, which is a prerequisite for endocytosis via the WT receptor (Fig. 10).

Toxin-induced receptor ubiquitination

The cytoplasmic tails of all isoforms of TEM8 and CMG2 contain lysine residues in numbers that vary from 3 in TEM8/2 to 14 in CMG2/4 and 16 in TEM8/1, all of which are potential ubiquitination sites. We found that anthrax PA triggers the ubiquitination of each of these receptors. Indeed, Lys-352 in TEM8/1 and the corresponding Lys-350 in CMG2/4 acquire this modification, and, thus, mutation of these lysines leads to the inhibition of PA endocytosis. Ubiquitination TEM8 is mediated by the E3 Ub ligase Cbl as shown by RNA silencing on HeLa cells. The modification is raft dependent because it occurred preferentially in DRMs and was inhibited by cholesterol extraction from the plasma membrane. Moreover, DRM association appears to be sufficient to induce ubiquitination because the AAAA TEM8/1 palmitoylation-deficient mutant is constitutively DRM associated as well as ubiquitinated.

The findings described in this study—i.e., that both palmitoylation and ubiquitination of TEM8 are important for anthrax toxin action—appear to be in disagreement with previous work showing that anthrax PA could be endocytosed in cells expressing a variety of COOH-terminal TEM8 truncation mutants (Liu and Leppla, 2003). In this latter study, all transmembrane-truncated TEM8 mutants, with the exception of the mutant lacking the entire cytoplasmic tail, retained palmitoylation and ubiquitination sites because they were all longer than TEM8 isoform 2. The completely tailless mutant was still able to internalize PA (Liu and Leppla, 2003). However, the conditions used were very different (higher PA concentrations and longer incubation times) than those in this study, and kinetics were not measured.

Concluding remarks

We have identified two posttranslational modifications of the anthrax toxin receptors, palmitoylation and ubiquitination, that have opposite effects on the uptake of receptors and play a crucial role in the mode of toxin action. Palmitoylation serves to spatially segregate the receptors away from their Ub ligase, which, in turn, controls their endocytosis and turnover.

In terms of anthrax toxin's mode of action, an intricate sequence of events can be envisioned (Fig. 10). PA binds to its receptors, and subsequent furin processing and heptamerization likely trigger a conformational change in the receptor, leading to its association with partner proteins and/or depalmitoylation. Altogether, this induces the redistribution of the toxin–receptor complex to lipid rafts, where an encounter with the E3 Ub ligase Cbl occurs. Ubiquitination of TEM8/CMG2 allows an interaction with proteins of the endocytic machinery harboring Ub-interacting domains (Hicke and Dunn, 2003). One of these is likely to be Eps15, which is involved in the formation of clathrin-coated pits, contains three Ub-interacting motifs (for review see Polo et al., 2003), and is required for anthrax toxin endocytosis (Abrami et al., 2003). Recruitment of the endocytosis machinery leads to rapid uptake and transport to early endosomes, where anthrax toxin receptor ubiquitination might play a second role in sorting into multivesicular bodies (Katzmann et al., 2002).

This study also illustrates a novel role for the palmitoylation of membrane proteins. It has recently been proposed that palmitoylation of the SNARE protein snf1 prevents recognition by the E3 ligases of critical lysines adjacent to the transmembrane domain by affecting protein conformation (Valdez-Taubas and Pelham, 2005). The absence of palmitoylation might also facilitate access of E3 ligase to lysines on TEM8/CMG2. However, more importantly, palmitoylation serves to spatially segregate these membrane proteins away from their Ub ligase, thus controlling their endocytosis.

Materials and methods

Proteins and antibodies

Anthrax toxin subunits (Leppla, 1988; Gordon et al., 1995) and aerolysin (Buckley, 1990) were purified as described previously. PA63 was generated by trypsin cleavage of PA83 (Abrami et al., 2003). Antibodies against PA (Liu and Leppla, 2003) and aerolysin (Fivaz et al., 2002) were polyclonals developed in our laboratories. Proteins and antibodies were obtained from the following companies: Anti NH_2-terminal MEK1 antibodies from Upstate Biotechnology; anti–caveolin-1 from Transduction Laboratories; anti-Ub (sc-8017) as well as antibodies and siRNA against human Cbl from Santa Cruz Biotechnology, Inc.; anti-HA coupled to beads, labeled, or unlabeled with HRP and anti-GFP from Roche; anti-V5 labeled or unlabeled with HRP from Invitrogen; HRP secondary antibodies from Pierce Chemical Co.; and FITC-conjugated secondary antibodies from Invitrogen.

Cells, plasmids, and transfections

BHK, HeLa, and anthrax toxin receptor–deficient CHO (here designated as $CHO^{\Delta ATR}$) cells were grown as described previously (Abrami et al., 2003, 2004; Liu and Leppla, 2003). The human CMG2 (isoform 4) gene tagged with a V5 epitope and cloned in the pcDNA3.1/ V5-HIS-TOPO expression vector was provided by J. Martignetti (Mount Sinai School of Medicine, New York, NY; Dowling et al., 2003). TEM8 isoforms 1 and 2 tagged with GFP and cloned in the pHS001-EGFP expression vector was provided by J. Young (Salk Institute, San Diego, CA; Rainey et al., 2005). Isoform 1 of human TEM8 gene tagged with an HA epitope was in the pIREShyg2 vector (Liu and Leppla, 2003).

Cysteine to alanine mutant constructs were generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) and were transfected into $CHO^{\Delta ATR}$ (1 μg cDNA/9.6-cm^2 plate) using Fugene (Roche). Stable lines were selected by two rounds of hygromycin resistance. Colonies were isolated by limited dilution. To silence Cbl, HeLa cells were transfected with 200 pmol/9.2-cm2 dish of siRNA using OligofectAMINE (Invitrogen) transfection reagent.

Toxin treatment and analysis

Confluent cells were incubated in incubation medium (IM; Glasgow minimal essential medium buffered with 10 mM Hepes, pH 7.4) at 4°C for 1 h with various combinations of proaerolysin, PA, and LF, washed, and placed at 37°C in IM for different times. Cells were lysed by incubation for 30 min at 4°C with radioimmunoprecipitation buffer (1% NP-40, 50 mM Tris-HCl, pH 7.4, 0.25% sodium deoxycholate, 150 mm NaCl, 1 mM EDTA, and a cocktail of protease inhibitors; Roche). Protein concentrations of extracts were determined with bicinchoninic acid (Pierce Chemical Co.). To convert surface PA^{7mer} to an SDS-resistant form, cell extracts were incubated at room temperature for 10 min with 145 mM NaCl and 20 mM MES-Tris, pH 4.5. SDS-PAGE was performed using 4–20% gels (NOVEX) under nonreducing conditions. Gels were transferred onto nitrocellulose as described previously (Abrami et al., 2004).

Drug and enzymatic treatments

To inhibit *N*-glycosylation, cells were treated with 10 μg/ml tunicamycin (Sigma-Aldrich) during the last 16 h of growth. For *N*-deglycosylation, cell extracts were boiled for 5 min with 1% SDS and 1% β-mercaptoethanol, diluted fivefold in 40 mM phosphate buffer, pH 7.0, containing 10 mM EDTA, 1% Triton X-100, 2.5 mM PMSF, and 1% β-mercaptoethanol, and were incubated for 16 h at 37°C with 10 U/ml *N*-glycosidase F. Endo H treatment was performed according to the manufacturer's instructions (New England Biolabs, Inc.). Palmitoylation was inhibited by pretreating cells with 100 μM 2-bromopalmitate (Sigma-Aldrich) for 1 h at 37°C . Chemical removal of S-palmitoylation was performed by treating cell extracts for 1 h at room temperature with 1 M hydroxylamine hydrochloride, pH 7.2.

Protein synthesis was blocked by a 30-min treatment with 10 μg/ml cycloheximide at 37°C. Endoplasmic reticulum–to-Golgi transport was blocked by 20 μg/ml brefeldin A pretreatment for 30 min at 37°C. The proteasome inhibitor MG132 (Sigma-Aldrich) was used at 10 μM during the 16 h in culture medium. To block lysosomal enzymes, cells were fed for 16 h with 250 μg/ml leupeptin. To extract cholesterol, cells were treated with 10 mM βMCD (Sigma-Aldrich) in IM for 30 min at 37°C, leading to a 59.3 ± 3.8% decrease in total cholesterol as measured by thin layer chromatography (Abrami and van der Goot, 1999).

Biochemical methods

DRMs were prepared using OptiPrep gradients as described previously (Abrami et al., 2003). Six fractions were collected from the top, and the total protein content of each fraction was precipitated with trichloroacetic acid (Abrami et al., 2003). For immunoprecipitations of TEM8 or CMG2, cells were lysed for 30 min at 4°C in immunoprecipitation buffer (0.5% NP-40, 500 mM Tris-HCl, pH 7.4, 20 mM EDTA, 10 mM NaF, 2 mM benzamidine, 1 mM *N*-ethyl-maleimide, and a cocktail of protease inhibitors), centrifuged for 3 min at 2,000 *g*, and supernatants were incubated for 2 h at 4°C with either HA-coupled agarose beads (Roche) or protein G–coupled beads (GE Healthcare) with 2 μg monoclonal antibody against V5. After washing of the beads, samples were boiled for 5 min under reducing conditions.

Radiolabeling experiments

To follow palmitoylation, TEM8/CMG2-expressing cells were incubated for 2 h at 37°C in IM with 200 μCI /ml ^{3}H-palmitic acid (9,10-^{3}H(N); American Radiolabeled Chemicals, Inc) , were washed, and submitted to immunoprecipitation. Beads were incubated for 30 min at 60°C in nonreducing sample buffer before SDS-PAGE. After fixation (25% isopropanol, 65% H_2O, and 10% acetic acid), gels were incubated for 30 min in enhancer Amplify NAMP100 (GE Healthcare), dried, and exposed to a Hyperfilm Multipurpose (GE Healthcare).

For metabolic labeling, CHO cells were transiently transfected for 30 h with TEM8/1-HA cDNAs, washed with methionine/cysteine-free medium, incubated for a 30-min pulse at 37°C with 50 μCi/ml [^{35}S]methionine/cysteine (Hartman Analytics), washed, and further incubated for different times at 37°C in complete medium with a 10-fold excess of nonradioactive methionine and cysteine. Receptors were immunoprecipitated and analyzed by SDS-PAGE.

Immunofluorescence microscopy

CHO cells were fixed with 3% formaldehyde, permeabilized with 0.1% Triton X-100, and labeled with anti-HA monoclonal antibodies followed by fluorescein isothiocyanate–conjugated goat anti–mouse IgG. Images were acquired using a 100× lens on a microscope (Axiophot; Carl Zeiss MicroImaging, Inc.) equipped with a cooled camera (model Orca; Hamamatsu) using the Openlab acquisition software (Improvision).

Online supplemental material

Three supplemental figures are provided. Fig. S1 shows that isoform 2 of TEM8, which has a very short cytoplasmic tail (Fig. 1 A), is palmitolyted. Fig. S2 shows that this isoform is also ubiquitinated. Finally, Fig. S3 shows than when toxin concentrations are adapted so that CCCC TEM8/1– and AAAA TEM8/1–expressing cells bind similar amounts of PA, MEK1 cleavage is observed in the former cells within an hour but not in the latter cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200507067/DC1.

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