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Evading the Proteasome: Absence of Lysine Residues Contributes to Pertussis Toxin Activity by Evasion of Proteasome Degradation▽

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Pertussis toxin (PT) is an important virulence factor produced by *Bordetella pertussis*. PT holotoxin comprises one enzymatically active A subunit (S1), associated with a pentamer of B subunits. PT is an ADP-ribosyltransferase that modifies several mammalian heterotrimeric G proteins. Some bacterial toxins are believed to undergo retrograde intracellular transport through the Golgi apparatus to the endoplasmic reticulum (ER). The ER-associated degradation (ERAD) pathway involves the removal of misfolded proteins from the ER and degradation upon their return to the cytosol; this pathway may be exploited by PT and other toxins. In the cytosol, ERAD substrates are ubiquitinated at lysine residues, targeting them to the proteasome for degradation. We hypothesize that S1 avoids ubiquitination and proteasome degradation due to its lack of lysine residues. We predicted that the addition of lysine residues would reduce PT toxicity by allowing ubiquitination and degradation to occur. Variant forms of PT were engineered, replacing one, two, or three arginines with lysines in a variety of locations on S1. Several variants were identified with wild-type in vitro enzymatic activity but reduced cellular activity, consistent with our hypothesis. Significant recovery of the cellular activity of these variants was observed when CHO cells were pretreated with a proteasome inhibitor. We concluded that the replacement of arginine residues with lysine in the S1 subunit of PT renders the toxin subject to proteasomal degradation, suggesting that wild-type PT avoids proteasome degradation due to an absence of lysine residues.

Whooping cough (or pertussis) is exclusively a human disease caused by an infection by the gram-negative coccobacillus *Bordetella pertussis*. The organism *B. pertussis* binds to ciliated cells of the respiratory tract (47). It produces a number of exotoxins: adenylate cyclase toxin, necrotizing toxin (dermonecrotic toxin), tracheal cytotoxin, and pertussis toxin (PT), the last toxin produced only by this bacterium. PT is an important virulence factor and exists as a 105-kDa protein with an AB_5 form. The holotoxin includes the B oligomer, which is organized into a pentameric ring composed of one S2, one S3, two S4, and one S5 subunit, which bind to as-yet-unidentified glycoconjugate receptors on the surface of the target cell, allowing for the internalization of the holotoxin by endocytosis (2, 50). The A subunit, also termed S1, bears enzymatic activity and is an ADP-ribosyltransferase that modifies heterotrimeric G proteins of the host cells, resulting in an inhibitory effect on G-protein-coupled signaling pathways (24, 29). PT has also been demonstrated to have a suppressive effect on the innate immune system in mice at early stages of infection (5, 7, 26). The exact role of PT in *B. pertussis* infection is currently under investigation, and thus, further study of the properties and trafficking methods of this toxin within the host cell should provide us with a better understanding of the organism and the infection.

A trafficking pathway for PT has been proposed based on the trafficking events of similar exotoxins, since the manner in which PT translocates within the mammalian cell in order to reach its target G proteins is still unresolved. It has been determined that PT holotoxin enters the cell by endocytosis (12, 51, 52). However, there is some evidence to suggest that several bacterial exotoxins are subject to retrograde transport by way of the Golgi apparatus to the endoplasmic reticulum (ER) (27, 37). There is some limited experimental evidence to support the subsequent retrograde transport of PT to the Golgi postinternalization by endocytosis. The cytotoxicity of PT has been shown to be inhibited by the treatment of mammalian cells with brefeldin A, a compound that disrupts the Golgi apparatus (17), and subcellular fractionation experiments have been performed in which PT was detected in this compartment (12, 51, 52). The trafficking of PT after its arrival in the Golgi has yet to be elucidated and is currently being investigated in our laboratory.

The ER-associated degradation (ERAD) pathway allows for the removal and degradation of misfolded proteins from the ER (3). This pathway involves the transport of substrates, in an unfolded form, out of the ER, possibly via the retrotranslocation pore-forming protein Derlin-1 (28, 55). There is accumulating evidence that other AB toxins (e.g., cholera toxin [CT], *Escherichia coli* heat-labile toxin, Shiga toxin, and ricin) traffic in a retrograde manner by way of the ERAD pathway (27). There is increasing evidence that other similar toxins exit the ER via the Sec61 translocon (32, 34, 35, 39, 41, 49, 56). Once ERAD substrates enter the cytosol, they are polyubiquitinated, the site of ubiquitination being lysine residues in the target protein. Ubiquitination is the signal for targeting of the substrate to the 26S proteasome, where degradation occurs (31). Since S1 (and the enzymatically active A subunit of other toxins) reaches target proteins located in the host cell cytosol, it must avoid proteasome degradation. However, there are also

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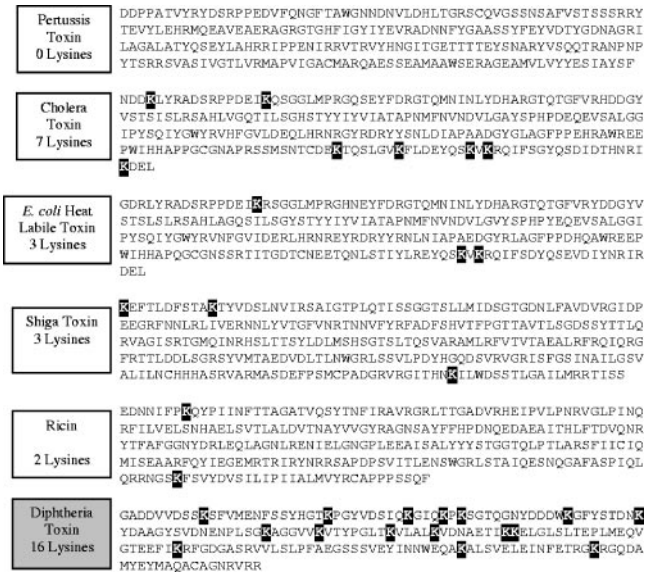

FIG. 1. Comparison of numbers of lysine residues in the active subunits of various exotoxins. The protein sequences of the active subunits of various AB toxins are listed, and lysine residues are highlighted in black. The A subunit of PT is distinguished by the absence of lysine residues, and the active subunits of other toxins that traffic via ERAD have a paucity of lysines, unlike diphtheria toxin, which has a large number of lysine residues and traffics by way of a different pathway.

examples of ubiquitin-independent proteasomal degradation (13, 40, 48). It is noteworthy that toxins that translocate out of the ER have very low numbers of lysine residues compared with toxins that traffic in other pathways (such as diphtheria toxin), and this is illustrated in Fig. 1. We hypothesize that the absence of lysine residues in the S1 subunit of PT allows the toxin to avoid ubiquitination and subsequent proteasomal degradation. Recently, it has been demonstrated that the addition of lysine residues to ricin and CT brings about their degradation by the proteasome and reduces their cytotoxicity (10, 11, 27).

Evidence for the passage of S1 from the ER to the cytosol was obtained previously by transfection of Chinese hamster ovary (CHO) cells with constructs encoding the S1 subunit of PT with a signal peptide to direct it to the ER (8). Wild-type and enzymatically inactive variant S1 constructs were engineered with and without the native bacterial signal peptide or a mammalian signal peptide. Stable transfectants were obtained in both CHO cells and Cos cells, as determined by indirect immunofluorescence. When coupled with a signal peptide, S1 was found to localize to the ER, whereas S1 with no signal peptide was found diffusely throughout the cell. The presence of the signal peptide did not inhibit the ability of S1 to ADP-ribosylate its target G proteins. The conclusion from these data was that S1 can traffic from the ER into the cytosol as previously hypothesized. However, the levels of S1 expression in these stable transfectants were significantly higher than the cell-associated levels observed when PT was applied to cells exogenously.

In this study, mutations were made at various locations on the S1 subunit, replacing arginine with lysine residues at one, two, and three positions. More than one lysine was substituted in a number of variants since it is possible that the signal or trigger for ubiquitination may require more than one lysine residue. The in vitro and cellular activities of these variant toxins were determined by an ADP-ribosylation assay. The effect of proteasome inhibitors on variant toxins was also determined in this manner.

MATERIALS AND METHODS

Growth conditions and bacterial strains. *B. pertussis* strains were grown on Bordet-Gengou agar (Difco) plates supplemented with 15% defibrinated sheep blood and, when necessary, antibiotics at the following concentrations: streptomycin, 400 μg ml^{-1}; nalidixic acid, 20 μg ml^{-1}; and gentamicin sulfate, 10 μg ml^{-1}. Alternatively, for liquid culture, strains were grown in Stainer-Scholte medium (42) supplemented with 1 g liter^{-1} heptakis-dimethylcyclodextrin (Sigma).

For cloning experiments, *E. coli* DH10B was used, and for conjugation with *B. pertussis*, the *E. coli* strain SM10 was used. These strains were grown either in LB broth containing 100 μg ml^{-1} ampicillin, if required, or on LB agar plates containing 10 μg ml^{-1} gentamicin.

The wild-type *B. pertussis* strain used in these experiments was an Str^r Nal^r derivative of W28 (Wellcome), and the strain bearing the enzymatically inactive toxin, denoted PT* (PT9K/129G), was constructed as previously described (33). The lysine variant toxins were derived as described below.

Construction of lysine variant toxins. The location of arginine residues for replacement with lysine residues was selected based on the crystal structure of PT (and S1 in particular) and the distance from the enzymatic core of the protein, such that the folding and function of the protein would probably not be affected due to the change in amino acid affecting the overall conformation. Substitution mutations were constructed by overlap extension PCR (21). The positions of the altered residues are listed in Table 1. Variant S1 was also constructed by replacing the arginine residues with alanine residues at positions 79 and 117, as a control, to confirm that the change in activity was due to the addition of lysine and not a loss of the arginine residues.

Purification of proteins. Proteins were purified from the cell culture supernatant by fetuin purification (25). The concentration of purified proteins was measured by a bicinchoninic acid assay (Pierce); then, 10% glycerol was added to the protein samples, which were stored at −80°C.

The terminal 20 amino acids of $G_i\alpha3$ protein fused to glutathione *S*-transferase (GST) (GST-αC20) were purified from a culture of *E. coli* DH10B containing the plasmid pGEX-αC20. Expression of the fusion protein was induced and then purified as previously described (7). Recovered proteins were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, Western blotting, and a bicinchoninic acid assay (Pierce) to determine the concentration.

In vitro ADP-ribosyltransferase assays. The substrate GST-αC20 was used as the target in an ADP-ribosylation assay modified from that of Xu and Barbieri (52). In a 25-μl final volume, the following reagents were added to a 1-μl aliquot of substrate: 0.1 M Tris HCl, pH 7.5, 20 mM dithiothreitol, 0.1 mM ATP, 0.1 μM [^{32}P]NAD (specific activity, 30 Ci mmol^{-1}; PerkinElmer), and 10 ng variant PT. Reagents were incubated at room temperature for 90 min, and then the reaction

TABLE 1. Summary of in vitro and cellular ADP-ribosyltransferase activities of PT with various arginine-to-lysine substitutions[a]

Strain	Position of mutation in S1	In vitro ADP-ribosylation activity (%)	Cellular ADP-ribosylation activity (%)
W28 (wild type)		100 ± 0	100 ± 0
PT* (control)	PT9K/R9G	0.97 ± 0.51	0 ± 0
R-to-A variant	R79A/R117A	117.2 ± 3.1	96.9 ± 0.4
11/47	R79K	116.1 ± 20.7	66.9 ± 0.45
11/68	R79K/R117K	101.4 ± 36.9	73.5 ± 6.04
12/26	R181K	101.0 ± 11.1	94.3 ± 6.1
14/42	R58K/R143K	91.1 ± 32.0	15.6 ± 9.5
14/37	R143K	111.8 ± 15.5	17.2
13/7	R79K/R117K/R181K	85.6 ± 2.8	2.9 ± 6.6

[a] Values are means ± standard deviations.

was halted by the addition of sample buffer. Reaction mixtures were loaded onto a 15% Tris HCl SDS gel (Bio-Rad). Postelectrophoretic separation gels were fixed for 30 min in fixer 1 (27% methanol, 4% acetic acid) and 30 min in fixer 2 (2% glycerol, 1 M sodium salicylate), then dried, and exposed to film.

Cellular ADP-ribosyltransferase assays. Near-confluent CHO cells treated exogenously with variant PT (for 3 h) or transfected CHO cells were harvested from 12-well plates by using a trypsin-like solution (TrypLE Express; Invitrogen). Recovered cells were washed in 1× phosphate-buffered saline to remove any media and then lysed by incubation on ice for 30 min in 0.5% Triton X-100 lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris HCl, pH 7.4, 0.01% NaN_3, and 0.5% Triton X-100). The resulting lysate was subjected to centrifugation at 13,000 rpm for 15 min; the supernatant was recovered (postnuclear supernatant [PNS]), transferred to sterile Eppendorf tubes, and stored at −20°C until required.

The resultant PNS was used in an ADP-ribosylation assay modified from that of Xu and Barbieri (52). In a 25-μl final volume, the following reagents were added to an aliquot (between 15 and 19 μl) of the PNS: 0.1 M Tris HCl, pH 7.5, 20 mM dithiothreitol, 0.1 mM ATP, 0.1 μM [^{32}P]NAD (specific activity, 30 Ci $mmol^{-1}$; PerkinElmer), and 10 ng PT. Reagents were incubated at room temperature for 90 min, and then the reaction was halted by the addition of sample buffer. Reaction mixtures were loaded onto a 15% Tris HCl SDS gel (Bio-Rad). Postelectrophoretic separation gels were fixed and dried as described above for the in vitro assay.

Band intensities from ADP-ribosyltransferase assays were determined by densitometry. These data were used to calculate the extent of ADP-ribosylation of target proteins by variant toxins compared to that by wild-type PT.

Proteasome inhibition assays. CHO or HeLa cells plated at 2.5×10^5 cells per well in 12-well plates were pretreated with either 20 nM *clasto*-lactacystin β-lactone (lactacystin; Sigma) or 10 nM epoxomicin (Sigma) and incubated for 1 h before cellular ADP-ribosyltransferase assays as described above.

Western blotting. Whole-cell lysate samples in loading buffer were run on 12% SDS-polyacrylamide gel electrophoresis gels and transferred onto either nitrocellulose or polyvinylidene difluoride membranes. For the detection of S1, filters were preincubated in blocking solution (5% nonfat milk powder in Tris-buffered saline–Tween 20) and then incubated with monoclonal antibody 1C7 or 2F2 specific to S1. This was followed by incubation with the secondary antibody peroxidase-conjugated anti-mouse immunoglobulin G (Amersham). Blots were detected using an enhanced chemiluminescence method (ECL Plus or ECL Advance; Amersham) and exposed to film (Biomax Light; Kodak).

RESULTS

Construction of variant toxins. A common property of S1 and the enzymatically active subunits of other toxins that traffic in an intracellular and retrograde manner is the low lysine content of these domains. S1, however, is unique among this group of toxins in that it contains no lysine residues. The important feature of these lysine residues is that they are targets for ubiquitination, a modification which occurs in the cytosol and targets proteins to the proteasome for degradation. Since we have hypothesized that PT S1 avoids proteasomal degradation by avoiding ubiquitination due to a lack of lysines, variant toxins were constructed by replacement of arginine residues with lysine residues on the S1 subunit.

To determine suitable locations for these substitutions, the crystal structure of S1 and the holotoxin were examined (43) in order to place substitutions at locations that are surface exposed on S1 and not located within the catalytic site of S1. Overlap extension PCR was used to generate substitutions at the required locations (Table 1), and constructs were introduced into *B. pertussis* by conjugation. A control construct was also generated where arginine residues at positions 79 and 117 were replaced with alanine residues, and this construct was used to confirm that the reduction in cellular activity is not due to the loss of arginine residues but to the presence of lysines. All of the variant toxins generated were efficiently secreted, as determined by Western blotting (data not shown).

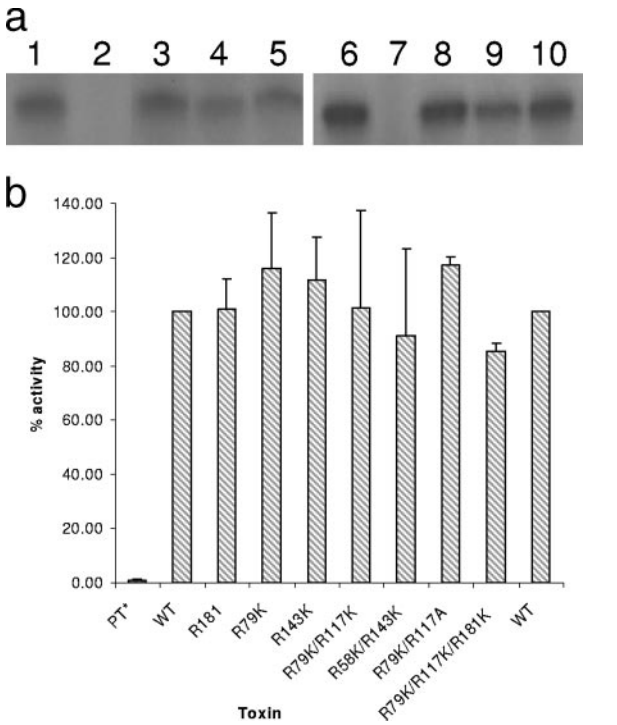

FIG. 2. (a) In vitro ADP-ribosylation analysis of various lysine variant PTs. Lysine variant proteins (1 nM) were incubated with purified G protein as per standard ADP-ribosylation protocol. Lanes: 1, wild-type PT (WT); 2, PT*; 3, R79K/R117K/R181K; 4, R58K/R143K; 5, R79K/R117K; 6, R143K; 7, PT*; 8, WT; 9, R79K; 10, R134K. Note that all variant toxins have activity comparable to that of wild-type PT. (b) Densitometry results of variant PT in vitro activity compared to that of wild-type PT. Analysis of in vitro ADP-ribosylation activity of variant PT toxins with one, two, or three lysine changes. Activity was measured by densitometry and compared to wild-type activity; data from three independent experiments are represented in this graph.

In vitro enzymatic activity of lysine variant PT toxins. The variant toxins were efficiently expressed and secreted in *B. pertussis*, as determined by Western blotting (data not shown). To confirm that the substitutions made in the variant PT molecules did not substantially affect the enzymatic activity of the proteins, in vitro ADP-ribosylation assays were conducted with the following variants: single substitutions R79K, R117K, R143K, and R181K, double substitutions R79K/R117K and R58K/R143K, and triple substitution R79K/R117K/R181K. Wild-type toxins in the form of both a commercially available source (Calbiochem) and our purified form (from W28) were used as positive controls, and the enzymatically inactive form of PT, termed PT* (PT9K/129G), was used as a negative control for the assay. All of the variants tested retained enzymatic activity at a level comparable to that of the wild-type toxin (Fig. 2), suggesting that the changes made to these variant proteins did not affect the enzymatic activity of the toxin. The activity of these variant toxins compared to that of the wild type was quantified by densitometry, using replicates from three independent in vitro ADP-ribosylation experiments. The results of this analysis are listed in Table 1; all toxins retained activity close to that of the wild type. The variant toxin with arginine-to-alanine substitutions at R79A and R117A (designed as a

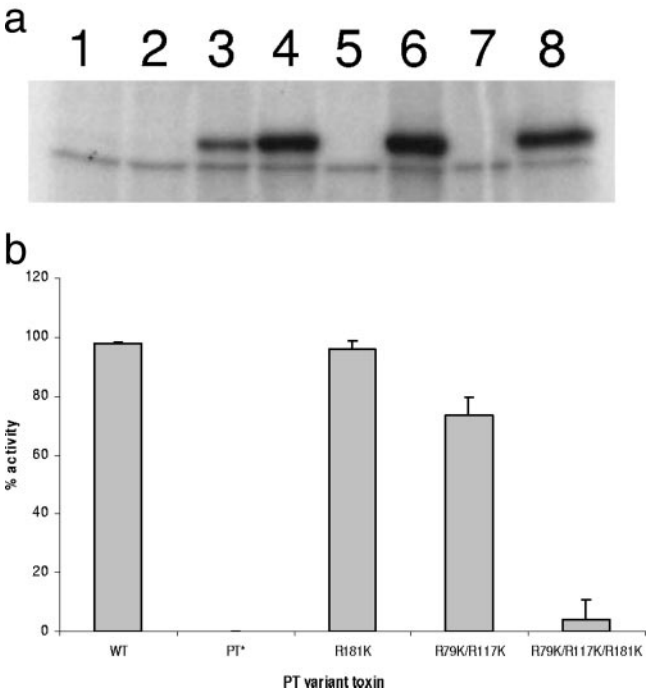

FIG. 3. (a) Cellular ADP-ribosylation analysis of CHO cells treated with lysine mutant PT. Lysine variant proteins were incubated with CHO cells and the PNS generated as per standard cellular ADP-ribosylation protocol. Lanes: 1, wild type PT [WT]; 2, 1K (R181K); 3, 2K (R79K/R117K); 4, 3K (R79K/R117K/R181K); 5, 1K (R79K); 6, PT*; 7, untreated CHO PNS without PT; 8, untreated CHO PNS with PT. The upper band (approximately 41 kDa) indicates ADP-ribosylated G proteins and thus a loss of activity of the variant toxin. The lower band is PT independent and is present in all lanes, including the control without PT. (b) Cellular ADP-ribosylation analysis of CHO cells treated with lysine mutant PT. Cellular ADP-ribosylation of G proteins in CHO cells following treatment with variant PTs with one, two, or three lysine substitutions. The assay was conducted as described in Materials and Methods; data from three independent experiments are represented. Statistical analysis by a *t* test indicated that the reductions in activity for the double and triple mutants were statistically significant: $P = 0.024$ and $P = 0.0015$, respectively.

control to illustrate that the observed effects of lysine substitutions are due to the addition of lysine residues and not the loss of arginine) also retained enzymatic activity comparable to that of the wild-type toxin (Table 1).

Cellular activity of variant PT toxins. Once retention of enzymatic activity had been confirmed in a number of variant S1 proteins, variant PT toxins were tested for activity in CHO cells by using a cellular ADP-ribosylation assay in order to investigate the activity of these variants within the mammalian cell. Near-confluent CHO cells were treated with 1 nM of variant or wild-type PT for 3 h at 37°C; cells were then lysed, and the PNS was recovered. The PNS was then used as the substrate in an in vitro ADP-ribosylation assay with wild-type PT. The results of these assays are illustrated in Fig. 3 and listed in Table 1. As expected, wild-type PT modified all available G proteins in the cells, demonstrated by an absence of a band from the in vitro assay (Fig. 3a, lane 1). PT* has no enzymatic activity, and thus, the G proteins in the cells remain unmodified and available for modification and labeling in the in vitro assay, resulting in a strong band (lane 6). The level of activity of the variant PT compared to that of the wild-type toxin differed depending upon the location and number of lysine substitutions. A reduction in ADP-ribosylation activity was observed to some extent in most of the variant toxins. There was a correlation between the number of lysine substitutions and the loss of enzymatic activity. The variant with three lysine substitutions had activity comparable to that of the inactive mutant PT*, suggesting that activity had been almost completely lost. Some toxins with a single lysine substitution had only a slight reduction in activity, and others such as the R143K variant had a more substantial loss of activity (Table 1). The control alanine variant retained cellular activity approaching that of the wild-type toxin, demonstrating that the loss of activity was due to the presence of lysines and not the loss of arginines. When the cellular activity of the alanine variant (R79A/R117A) was compared to that of the equivalent lysine variant (R79K/R117K), the effect of lysine substitution was further illustrated, since the alanine variant had 96.9% activity compared to 73.5% activity in the lysine variant. Several variants have been identified that have in vitro enzymatic activity equivalent to that of the wild type but reduced cellular activity (Table 1).

Inactivation of the proteasome restores lysine variant PT cellular activity. We hypothesized that if the loss in activity for the variant S1 proteins is due to degradation by the proteasome, treating CHO cells with the variant proteins in the presence of a proteasome inhibitor would result in the recovery of cellular activity as determined by ADP-ribosylation. CHO cells were pretreated for 1 h with a proteasome inhibitor and then treated with exogenously applied variant PT as described for cellular ADP-ribosylation experiments. The PNS was harvested and cellular ADP-ribosylation assays performed. Treatment of CHO cells with an irreversible proteasome inhibitor, lactacystin, resulted in partial recovery of activity (Fig. 4a). Lactacystin irreversibly inhibits the chymotrypsin- and trypsin-like peptidase activities of proteasomes (16). Another proteasome inhibitor, epoxomicin, was tested to ensure that the observed effect is not due to a property of lactacystin other than inhibition of the proteasome, and similar results were observed (data not shown).

The variant PT proteins with more than one lysine change had significantly greater cellular activity when host cells were pretreated with proteasome inhibitor. The results of this analysis (based on the average from five independent experiments) are summarized in Fig. 4b, where representatives of the variant toxins with one, two, or three lysine changes are shown. The variant with only a single lysine change exhibited activity approaching that of the wild type in untreated CHO cells. The activity of the two-lysine variant was increased more than 2-fold in the presence of the proteasome inhibitor, and the triple-lysine variant exhibited more than an 11-fold increase in activity upon the addition of the proteasome inhibitor. (In some experiments, the activity of the wild-type toxin is slightly reduced in the presence of lactacystin. This effect further emphasizes the increase in activity observed with lysine variant toxins when treated with proteasome inhibitor, since they are also able to overcome this slight inhibitory effect encountered by the wild type.)

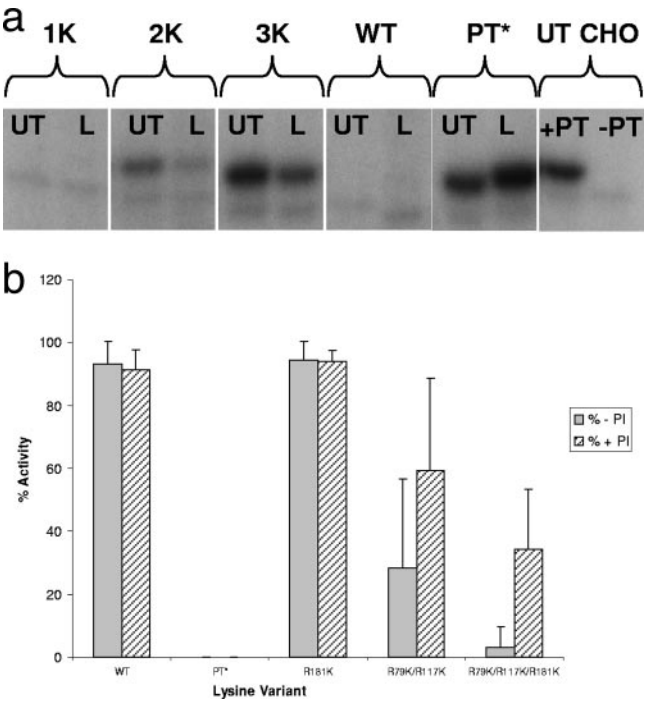

FIG. 4. (a) Inactivation of the proteasome with the inhibitor lactacystin allows for the recovery of lysine variant PT activity, as determined by cellular ADP-ribosylation. Cellular ADP-ribosylation analysis of CHO cells treated with variant PT toxins in the presence and absence of the proteasome inhibitor. UT indicates no proteasome treatment; L indicates treatment with 20 nM lactacystin. 1K, R181K; 2K, R79K/R117K; 3K, R79K/R117K/R181K; WT, wild-type S1 (W28); PT*, enzymatically inactive PT. Control ADP-ribosylation reactions were performed using the PNS from CHO cells without prior PT treatment, which was incubated with (+PT) or without (−PT) PT in the in vitro step of the assay. The UT CHO +PT lane illustrates the band intensity for untreated CHO cells. The UT CHO −PT lane illustrates that the lower band represents a PT-independent event. (b) Effect of proteasome inhibitor on the activity of lysine variant PT in CHO cells, as determined by densitometry. Cellular ADP-ribosylation of CHO cells treated with lysine variant PTs in the presence and absence of proteasome inhibitor. Data from five independent experiments are summarized in this figure. Data were analyzed for statistical significance by a *t* test, and the recovery of activity for the triple variant upon the addition of proteasome inhibitor was significant: $P = 0.003$.

DISCUSSION

As predicted in our hypothesis, the replacement of arginine residues with lysine residues renders the PT S1 subunit subject to degradation by the proteasome, presumably due to the presence of a site(s) for ubiquitination. The enzymatic activity of lysine variant toxins is relatively unaffected in vitro, as demonstrated by the retention of ADP-ribosyltransferase activity. However, when these variant PT molecules are exogenously applied to mammalian cells, thus entering and trafficking through the cell to its target G proteins (presumably in a retrograde manner), their activity is significantly reduced and, in the case of the triple-lysine variant, reduced to levels approaching that of the enzymatically inactive mutant PT*. From these observations, we can conclude that a single lysine substitution can be sufficient to reduce the cellular activity of S1. The alteration of multiple arginine residues to lysine generally causes a greater reduction in the cellular activity of S1 than a single residue change. It can also be noted that not only the number of lysine residues substituted but also the location of this lysine substitution has various effects on the extent of reduction of S1 activity. Perhaps some of the lysines substituted into S1 are protected by their location within the protein, and upon exiting the ER, S1 is able to partially fold, protecting what would otherwise be exposed lysine residues available for ubiquitination. The control variant in which two arginine residues were replaced with alanine residues had in vitro activity equivalent to that of the wild type and cellular activity approaching that of the wild type, further suggesting that the presence of lysine rather than the absence of arginine was the cause of the loss of activity and consequent degradation.

Having demonstrated that the replacement of arginine residues with lysine brings about a reduction in cellular activity, we generated evidence that strongly suggests that the loss of S1 activity observed in these variants was due to proteasomal degradation, presumably brought about by the introduction of a site(s) for ubiquitination and hence targeting to the proteasome. When CHO cells were treated with an irreversible proteasome inhibitor prior to treatment with exogenously applied variant PT, a recovery in cellular activity, as determined by an ADP-ribosylation assay, was observed. Some variants with single lysine changes exhibited activity approaching that of wild-type S1. At least twofold recovery of activity was seen in most variant S1 toxins in the presence of proteasome inhibitors. The greatest effect was observed with the triple-lysine variant, which had little cellular activity; however, upon the addition of the proteasome inhibitor, this toxin yielded significant cellular activity. Incomplete recovery of activity in proteasome inhibition experiments may have been due to only partial inactivation of the proteasome, resulting in some residual degradation activity. Evidence for this incomplete inhibition of degradation in some well-characterized ERAD substrates, e.g., the cystic fibrosis transmembrane conductance regulator, has previously been observed (18, 23).

Alternatively, the ubiquitination of the variants alone could alter the enzymatic activity of the toxin, either by a conformational change or by a change in its ability to traffic via usual pathways and hence reach target G proteins. This incomplete recovery of cellular activity may be a result of lysine variant toxins exiting the ER less efficiently than wild-type S1. Until we have a reagent that inhibits the exit of PT from the ER, we cannot completely define the trafficking pathway of PT in the mammalian cell.

The hypothetical trafficking pathway of PT has yet to be completely elucidated, and some of this pathway is based upon evidence derived from work with other exotoxins with similar structures, such as *E. coli* heat-labile toxin, Shiga toxin, CT, and the plant toxin ricin. Hazes and Read (20) proposed an intracellular trafficking pathway for PT implicating the exploitation of the ERAD pathway. This model proposed that PT binds to cell surface receptors and that the holotoxin is internalized by endocytosis in a cytochalasin D-independent manner (12, 38, 51, 52). The work conducted in our study begins to address the events upon S1 entry into the cytosol.

The paucity of lysine residues in the AB_5 exotoxins thought to traffic in a retrograde manner has been noted as a feature that may allow them to avoid targeting to the 26S proteasome

and consequent degradation by this structure once they have been translocated into the cytosol from the ER, since lysine residues are sites for ubiquitination, the signal for targeting proteins to the proteasome (20). However, the proteasome is also able to recognize and degrade unubiquitinated proteins that are in an unfolded state (46). It has been demonstrated for CT that proteolysis of a fraction of the CT subunit A1 (CTA1) pool occurs despite a low number of lysine residues, suggesting a ubiquitin-independent mechanism of degradation (44). In the case of CT, it is likely that the paucity of lysine residues in combination with the rapid refolding of CT, which occurs upon entry into the cytosol, allows the toxin to reach its target G proteins and avoid proteasomal degradation in the cytosolic compartment (36). Recently, a ubiquitin-independent degradation pathway for S1 has been described, suggesting that the thermostability of S1 may have a significant effect on the retrotranslocation of this molecule (30). A model has been proposed whereby the heat-labile nature of the A subunits of some AB toxins (such as CT and ricin) is connected to both their translocation and degradation (45). S1 is a heat-labile protein, and its secondary and tertiary structures are disturbed at physiological temperatures, unlike the holotoxin and PT B oligomer, which are stable at this temperature. Studies suggest that once S1 has become dissociated from the B oligomer in the ER, it unfolds due to its temperature instability, which triggers ERAD and allows for the degradation of unfolded S1 by the 20S proteasome in a ubiquitin-independent mechanism (30). However, sufficient refolding of S1 must occur to allow activity in the cytosol.

It is interesting to note that while many of these exotoxins share similarities in structure and aspects of their retrograde trafficking pathways, there are some slight differences in their fate upon exit from the ER into the cytosol. The activity of wild-type CT is not enhanced by the addition of proteasome inhibitors (36); in contrast, the activity of wild-type ricin is enhanced in the presence of proteasome inhibitors (10). Ricin has a paucity of lysines, and a fraction of ricin is more efficiently targeted to the proteasome than is CT (10). It is possible that ricin is unable to refold as quickly as CT, exposing ricin posttranslocation to conditions favorable to polyubiquitination and proteasomal degradation for longer periods. Our results suggest that PT is more like CT than ricin in that the addition of proteasome inhibitors does not enhance the activity of wild-type PT and, in fact, a slight inhibition of activity is observed. Changing the number of lysine residues in these AB toxins has had similar effects in that the cytotoxicity of the variant toxin is reduced compared to that of the wild-type toxin since the variant has become a substrate for proteasomal degradation. The addition of lysines to the CTA1 chain brought about moderate degradation by the proteasome (36). Both CT and ricin differ from PT in that they contain some lysine residues. The replacement of lysines at two positions in CT with arginines had no effect on activity. The N-terminal region of CT is a potential target for ubiquitination, but blocking of this potential site by carbamylation also had no effect on the toxicity (36). Wild-type PT presumably folds rapidly in the cytosol, avoiding ubiquitin-independent proteasomal degradation.

Lysine residues were added to CT to examine their effect on degradation by substitution; reduced activity was observed in these variants to various degrees. This loss in activity could be recovered in all variant toxins by the addition of proteasome inhibitors (36). Interestingly, the defect in activity seen with the substitution of three lysine residues in PT was much more dramatic than the activity loss seen with CT and ricin variants.

Ricin, an rRNA *N*-glycosidase, reaches the ER only at low levels (15), and it has been shown that this toxin is not a substrate for ubiquitination (19). As seen in CT, the two lysine residues in wild-type ricin toxin A subunit are not effective substrates for ubiquitination; the replacement of these lysine residues with arginines yielded the same activity as that of the wild type (10). A defect in cytotoxicity was observed in variant ricin toxin A subunit with two lysine substitutions, while four lysines substitutions resulted in an even greater defect. The toxicity of these variants was recovered upon the addition of a proteasome inhibitor (10).

Recently, evidence for the exploitation of the ERAD pathway by AB toxins has been obtained by the association of p97 with these substrates. The AAA ATPase p97 is also known as valosin-containing protein and is involved in the retrotranslocation of ERAD substrates (4, 9). Although its exact role is unknown, it is thought that p97 may pull substrates out of the translocation pore and into the cytosol (22, 53). The cytotoxicity of CT, ricin, and *Pseudomonas aeruginosa* exotoxin A is inhibited in p97 mutant cell lines; in contrast, diphtheria toxin toxicity has been shown to be unaffected by this mutant (1). A role for p97 in retrotranslocation is further implied by its association with several other proteins involved in this pathway, such as the pore-forming protein Derlin-1, Ufd1 (a ubiquitin fusion degradation protein), and Npl4, which has an undefined role in the recognition and targeting of ubiquitinated proteins to the proteasome. If p97 is indeed involved in the translocation of ERAD proteins, the fact that ubiquitination is not essential for the binding of p97 to these substrates (14, 54) may be of importance to AB toxins that are poor subjects for ubiquitination due to their lysine paucity, particularly PT S1. p97 has been shown to associate with CTA in Vero cells, as determined by immunoprecipitation; these factors were found to coprecipitate, which suggests that their interaction occurs in the cytosol (1).

We found that indirect immunofluorescence analysis of CHO cells transfected with the lysine variant toxins further illustrated that the addition of lysine residues rendered S1 subject to proteasomal degradation, since an increase in variant S1 expression was observed in transfectants upon the addition of a proteasome inhibitor (data not shown). In transfected cell lines, it was surprising to find that undetectable levels of variant PT (at least at the sensitivity limits of Western blot analysis) can still result in high levels of activity; this is at odds with data obtained with exogenously applied variant PT, where a reduction in activity was observed due to the addition of lysine residues and may be due to the very low level of S1 that reaches cytosolic targets.

We hypothesize that PT traffics in a retrograde manner from the Golgi to the ER and subsequently from the ER to the cytosol. Retrograde trafficking of S1 to the ER was indicated previously in studies using an enzymatically inactivated form of PT (PT9K/129G) as a delivery vehicle for cytolytic T-lymphocyte epitopes to major histocompatibility complex class I (6). It was hypothesized that the delivery pathway used by the fusion protein may be the normal trafficking pathway of PT. Treat-

ment of cells with brefeldin A (a reagent that disrupts the structure of the Golgi) prevented the presentation of epitopes, suggesting that the fusion protein traffics via the Golgi, consistent with a retrograde pathway. The fusion protein was hypothesized to reach the ER, where proteolytic release of the epitope occurred. Epitope delivery to major histocompatibility complex class I was unaffected by proteasome inhibition, indicating that the fusion protein was not processed in the cytosol, probably due to a lack of ubiquitination and proteasome targeting (6). Evidence for the trafficking of PT holotoxin to the Golgi and ER has recently been obtained in our lab by generating PT constructs tagged with peptides for a Golgi-specific activity (tyrosine sulfation) and an ER-specific activity (N-glycosylation) (R. D. Plaut and N. H. Carbonetti, unpublished data).

We have demonstrated that the replacement of surface-exposed arginine residues in PT S1 with lysine residues renders the toxin subject to degradation by the proteasome, presumably by ubiquitination of the lysine residues; these observations are similar to those found for other toxins such as CT and ricin. Attempts to detect ubiquitinated species of variant PT by immunoprecipitation were unsuccessful, most likely due to the low level of toxin within the cells. A greater reduction in toxicity is seen in variants with more lysine substitutions.

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