Modulation of the intracellular stability and toxicity of diphtheria toxin through degradation by the N-end rule pathway

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The enzymatically active A-fragment of diphtheria toxin enters the cytosol of sensitive cells where it inhibits protein synthesis by inactivating elongation factor 2 (EF-2). We have constructed a number of diphtheria toxin mutants that are degraded by the N-end rule pathway in Vero cells, and that display a wide range of intracellular stabilities. The degradation could be inhibited by the proteasome inhibitor lactacystin, indicating that the proteasome is responsible for N-end rule-mediated degradation in mammalian cells. Previously, the N-end rule has been investigated by studying the co-translational degradation of intracellularly expressed β -galactosidase. Our work shows that a mature protein entering the cytosol from the exterior can also be degraded by the N-end rule pathway with a similar, but not identical specificity to that previously found. We found a correlation between the intracellular stability of the mutants and their toxic effect on cells, thus demonstrating a novel manner of modulating the toxicity of a protein toxin. The data also indicate that the inactivation of EF-2 is the rate-limiting step in the intoxication process.

Keywords: diphtheria toxin/FLAG epitope/N-end rule/protein degradation/Vero cells

Introduction

Proteins that are destined for degradation within the cell usually contain specific degradation signals, degrons. The N-end rule degron (reviewed in Varshavsky, 1995a, 1996) which relates the *in vivo* half-life of a protein to its amino-terminal residue, was the first to be discovered, initially in yeast (Bachmair *et al.*, 1986). When fusions (denoted Ub–X– β -gal) between ubiquitin (Ub) and β -galactosidase (β -gal) were expressed in yeast, the N-terminal ubiquitin moiety was rapidly cleaved off, except when the amino acid, X, at the junction was proline. This provided a means of generating otherwise identical X– β -gal proteins with different N-terminal amino acids, and it turned out that the different X– β -gal proteins were either long-lived or rapidly degraded when expressed in yeast, depending on the identity of the N-terminal amino acid (Bachmair *et al.*, 1986).

The N-end rule pathway has since been found to exist both in bacteria (Tobias *et al.*, 1991) and in higher eukaryotes (Gonda *et al.*, 1989; Levy *et al.*, 1996). According to studies with X– β -gal proteins *in vitro* in

reticulocyte lysates (Gonda *et al.*, 1989) and *in vivo* in L-cells (Levy *et al.*, 1996), destabilizing amino acids can be divided into three groups in mammalian cells: (i) positively charged residues (Lys, Arg, His); (ii) bulky, hydrophobic residues (Phe, Tyr, Trp, Leu); and (iii) some small, uncharged amino acids (Thr, and possibly Ala, Ser). It also appears that the three groups of unstable proteins are recognized by three distinct activities, since the degradation of X– β -gal *in vitro* in a reticulocyte lysate can be inhibited by a short peptide with an N-terminal amino acid belonging to the same group as X, but not by a peptide with an N-terminal amino acid from a different group (Gonda *et al.*, 1989). When the N-terminal amino acid is Cys, Glu or Asp, an Arg residue is added N-terminally (Ferber and Ciechanover, 1987; Gonda *et al.*, 1989), thereby targeting the proteins for degradation, and these amino acids are thus referred to as secondary destabilizing residues. Furthermore, N-terminal Gln or Asn residues can be converted by deamidation to Glu and Asp, respectively, and are therefore denoted tertiary destabilizing amino acids (Gonda *et al.*, 1989; Baker and Varshavsky, 1995; Grigoryev *et al.*, 1996).

Diphtheria toxin is one of several bacterial and plant toxins that act enzymatically on intracellular targets. Active diphtheria toxin consists of two fragments, A (21 kDa) and B (37 kDa) (Drazin *et al.*, 1971), linked by a disulfide bond (Collier and Kandel, 1971). The B-fragment is responsible for binding of the toxin to specific receptors on the surface of sensitive cells (Uchida *et al.*, 1972), and it is also involved in the translocation of the A-fragment to the cytosol. The A-fragment catalyses the inactivation of elongation factor 2 (EF-2) through ADP-ribosylation, leading to inhibition of protein synthesis (Honjo *et al.*, 1971) and cell death (Yamaizumi *et al.*, 1978). Upon receptor binding, the toxin is endocytosed and the low pH in the endosomes causes unfolding of the toxin molecule (Blewitt *et al.*, 1985), thereby inducing the translocation of the A-fragment to the cytosol (Sandvig *et al.*, 1984). If cells with surface-bound diphtheria toxin are briefly exposed to buffer of low pH, thereby mimicking the conditions in the endosome, direct translocation of the A-fragment across the plasma membrane is induced (Draper and Simon, 1980; Sandvig and Olsnes, 1980).

We discovered that when a short peptide, the FLAG epitope (Hopp *et al.*, 1988), was fused N-terminally to the diphtheria toxin A-fragment, the resulting protein was highly unstable upon translocation to the cytosol. The N-terminal amino acid of this protein is Asp, which is a destabilizing amino acid residue according to the N-end rule, and we have systematically replaced the N-terminal Asp residue of this protein with the remaining amino acids (except Pro) and studied the intracellular stability of the resulting mutant proteins. Previous studies on the specificity of the N-end rule *in vivo* have investigated the

co-translational degradation of intracellularly expressed X- β -gal (Bachmair *et al.*, 1986; Levy *et al.*, 1996), and we were interested in studying the specificity of the N-end rule using a fundamentally different model protein which enters the cell from the outside via a receptor-dependent mechanism and is therefore mature when it encounters the degradation machinery. Also, we were interested in studying how destabilization of the toxin A-fragment would influence its toxicity.

Results

Description of mutant toxins and characterization of their *in vivo* stability

The background for this study is the following serendipitous observation. To facilitate purification of the diphtheria toxin mutant E148S (Wilson *et al.*, 1990) upon expression in *Escherichia coli*, we fused an octapeptide, the FLAG peptide (Hopp *et al.*, 1988), to the N-terminus of this protein. The FLAG peptide consists of the sequence Asp-Tyr-Lys-Asp, which is recognized by the anti-FLAG M1 antibody (Kodak IBI, New Haven, CT), followed by the tetrapeptide Asp-Asp-Asp-Lys, a recognition motif for enterokinase. The toxin was expressed in *E.coli* and purified on an anti-FLAG affinity column, but we were unable to cleave off the FLAG peptide with enterokinase. When the purified toxin was labelled with ^{125}I , nicked with trypsin, bound to the surface of Vero cells and translocation of the A-fragment to the cytosol was induced by a brief exposure to low pH medium, the protein proved to be unstable in the cytosol (data not shown).

Since the fusion between the diphtheria toxin A-fragment and the FLAG peptide contained an N-terminal Asp residue, which is destabilizing according to the N-end rule in eukaryotic cells, we considered it likely that the protein was degraded by the N-end rule pathway. We reasoned that the specificity of the N-end rule in living mammalian cells could be studied by systematically replacing the N-terminal Asp residue of this protein with other amino acids. However, the purification of diphtheria toxin mutants on the anti-FLAG affinity column did not work reproducibly, and since the rabbit reticulocyte lysate has proven to be a reliable system for the expression of diphtheria toxin, we decided to use this expression system instead.

A protein expressed in a reticulocyte lysate is initiated with a Met residue, and to make a fusion protein between the FLAG peptide and the wild-type diphtheria toxin A-fragment, with any amino acid, X, at the N-terminus, a recognition site for a site-specific protease, factor Xa, was placed immediately upstream of the FLAG peptide, and the resulting protein was denoted pre-X-FLAG-DTA (Figure 1). Cleavage of the protein with factor Xa renders the amino acid X N-terminal, thereby generating X-FLAG-DTA, and the ^{125}I -labelled protein used in the initial experiments described above thus has an N-terminal sequence identical to that of Asp-FLAG-DTA. Using a PCR-based approach, we constructed plasmids encoding the 20 different pre-X-FLAG-DTA proteins. The plasmids were transcribed *in vitro* from a T3 promoter, followed by translation of the mRNA in a rabbit reticulocyte lysate. To generate radiolabelled mutant toxin, the A-fragment was translated in a reticulocyte lysate in the presence

Fig. 1. Scheme of constructs used. A precursor, pre-X-FLAG-DTA (X = any amino acid except Pro), containing a factor Xa recognition sequence (underlined), was cleaved with factor Xa, thereby generating X-FLAG-DTA. When X = Asp, the N-terminal octapeptide (double underlined) of X-FLAG-DTA corresponds to the FLAG peptide (Hopp *et al.*, 1988). A mutant protein, denoted Phe-DTA, where amino acids 2–189 of the diphtheria toxin A-fragment were preceded by the tripeptide Phe-Ser-Gly, was also generated by cleaving a precursor form, pre-Phe-DTA, with factor Xa. Upon secretion of the toxin from *Corynebacterium diphtheriae*, Gly is the N-terminal amino acid of the A-fragment, but for *in vitro* expression of the toxin, a form of the A-fragment with an N-terminal Met residue was used, here denoted WT.

of [^{35}S]methionine, mixed with a translation mixture containing unlabelled B-fragment (DTB), followed by dialysis to remove reducing agents, and thereby facilitating the formation of the holotoxin (pre-X-FLAG-DTA+DTB) through disulfide bond formation. Subsequently, the mixture was treated with factor Xa, generating X-FLAG-DTA+DTB. All of the 20 different proteins could be efficiently cleaved by factor Xa, except when X = Pro where no cleavage was observed (data not shown).

To study the intracellular stability of an X-FLAG-DTA protein, the holotoxin, X-FLAG-DTA+DTB, was bound to the surface of Vero cells, which were then briefly exposed to a buffer of pH 4.8, leading to translocation of the A-fragment to the cytosol with a concomitant reduction of the disulfide bond joining the A- and B-fragments. Subsequently, the cells were incubated in growth medium at 37°C for varying time periods and the radiolabelled A-fragment present in the cells was analysed by SDS-PAGE. In the initial experiments with Vero cells, we found that when X = Phe, Tyr, Trp, Asp, Asn, Glu, Gln, Lys, Arg, His, the X-DTA-FLAG proteins were highly unstable, with half-lives in the range 10–30 min, while the remaining mutants were considerably more stable, with half-lives between 3 h and >12 h (data not shown). However, since wild-type Vero cells are eventually killed by the toxin, we chose in later experiments to use a mutant Vero cell line, Vero-Dr22, which is resistant towards the intracellular action of the toxin but is still able to translocate it to the cytosol (Sundan *et al.*, 1982; Wiedlocha *et al.*, 1994). The results obtained with the Vero-Dr22 cells were qualitatively very similar to those obtained with wild-type Vero cells, although the half-lives of the short-lived mutants were generally somewhat longer. Representative degradation patterns are shown in Figure 2A, and the degradation of

Fig. 2. *In vivo* degradation of mutant A-fragments in Vero-Dr22 cells. *In vitro*-translated, [35 S]methionine-labelled toxin, reconstituted from separately expressed A- and B-fragments, was added to Vero-Dr22 cells, which were incubated for 20 min at room temperature. Unbound toxin was washed away, and the cells were incubated for 3 min at 37°C in MES-gluconate buffer, pH 4.8, to induce A-fragment translocation, followed by incubation in growth medium for various time periods at 37°C. Finally, the cells were lysed, and the TCA-precipitable material was analysed by non-reducing SDS-PAGE and fluorography or phosphorimaging. (A) Typical degradation patterns for different X-FLAG-DTA proteins. (B) Degradation curves for rapidly degraded mutants. The bands were quantitated by scanning densitometry of X-ray films. All the different mutants were analysed in parallel in the same experiment and each curve represents the average of three independent experiments. (C) Degradation curves for slowly degraded mutants. The bands were quantitated by phosphorimaging and each curve represents the average of three different experiments. In the figure pre-Asp-Flag-DTA has been abbreviated to preAsp. (D) 24 h time point for long-lived proteins. The error bars represent standard deviation in triplicate experiments.

these proteins was complete, without any visible intermediates (data not shown). The relatively unstable mutants (X = Phe, Tyr, Trp, Asp, Asn, Glu, Gln, Lys, Arg, His) displayed half-lives between 0.2 h and 1.6 h (Figure 2B; Table I), while the remaining, relatively stable mutants had half-lives from 3 h to >12 h (Figure 2C; Table I). When X = Glu, a small decrease in the migration rate was observed at later time points compared with the initial rate (Figure 2A, upper panel), and a similar decrease was observed in some, but not all, experiments when X = Gln or Asn (data not shown). A likely explanation for this change in migration rate is that Glu, Gln and Asn are secondary and tertiary destabilizing residues, and the slower-migrating form represents the N-terminally arginylated protein.

In these experiments, we also included the wild-type A-fragment, denoted WT (Figure 1), which was one of the most stable proteins tested. Diphtheria toxin secreted from bacteria contains Gly at the N-terminus of the A-fragment, but to facilitate expression of the toxin in a reticulocyte lysate, the wild-type A-fragment we used contained Met at the N-terminus, a substitution that does not affect toxin activity (Stenmark *et al.*, 1992). We also carried out experiments with the uncleaved precursor form of the protein where X = Asp, pre-Asp-FLAG-DTA (abbreviated preAsp) which was very stable (Figure 2C and D; Table I). In the case of the most stable mutants (WT, preAsp, X = Val, Met, Gly, Cys, Ile) we also performed an experiment where the cells were incubated

for 24 h upon translocation of the A-fragment to the cytosol. We found that the three proteins with an N-terminal Met (WT, preAsp, Met-FLAG) were the most stable, with 40–50% of the intact protein remaining after 24 h, whereas for the others (Val, Gly, Ile, Cys) the corresponding value was 5–20% (Figure 2D).

We also constructed a mutant A-fragment, denoted Phe-DTA, which was generated by the cleavage of a precursor, pre-Phe-DTA, with factor Xa (Figure 1). It represents a protein where the initial methionine of the wild-type A-fragment, WT, has been replaced by the tripeptide Phe-Ser-Gly. Notably, Phe-DTA displayed a half-life of 3.3 h, being considerably more stable than Phe-FLAG-DTA, which had a half-life of 0.5 h (Figure 2B and C; Table I). A previous study showed that the intracellular half-life of an N-end rule substrate could be substantially influenced by varying the distance between the N-terminal residue and downstream lysine residues, which can work as ubiquitin attachment sites (Bachmair and Varshavsky, 1989). Therefore, a likely explanation for the difference in stability between the two mutants with N-terminal Phe, is that the longer extension in the case of Phe-FLAG-DTA may generate a particularly favourable distance between the N-terminal residue and an internal lysine residue, or alternatively, that one or both of the two lysine residues in the FLAG peptide can function as ubiquitinylation sites.

In previous *in vivo* studies of the N-end rule pathway, the co-translational degradation of intracellularly expressed

Table I. Half-life and toxicity of X-FLAG-DTA proteins

	Vero-Dr22 cells (h)	Reticulocyte lysates (h)	IC ₅₀ (pM)
Glu	0.24 ± 0.03	4	6.48
Gln	0.30 ± 0.16	4	1.73
Trp	0.35 ± 0.12	1	3.84
Phe	0.51 ± 0.10	1	2.62
Asn	0.57 ± 0.07	4	3.98
Arg	0.83 ± 0.17	1	4.35
Lys	0.90 ± 0.17	1	2.80
His	1.08 ± 0.25	1	0.86
Tyr	1.46 ± 0.36	4	1.22
Asp	1.61 ± 0.28	4	1.03
Ala	2.9 ± 0.7	>>4	0.77
Phe-DTA	3.3 ± 1.2	>>4	1.05
Ser	3.5 ± 0.6	>>4	1.80
Leu	4.5 ± 0.5	>>4	0.80
Thr	6.2 ± 1.3	>>4	0.88
Val	7.8 ± 1.1	>>4	0.49
Ile	9.8 ± 0.6	>>4	0.78
Gly	9.8 ± 0.7	>>4	0.88
Cys	11.1 ± 1.8	>>4	1.32
Met	19.3 ± 5.1	>>4	0.92
WT	26.5 ± 4.8	>>4	0.30
preAsp	28.8 ± 6.6	>>4	0.65

Based on the data used to construct the curves in Figure 2B and C, the *in vivo* half-life of the various mutants was calculated, assuming first-order kinetics of degradation. In the case of the most stable proteins the 24 h time points from Figure 2D were merged with the data from Figure 2C, generating hybrid curves that were used to calculate the half-lives. For each of the three sets of data the half-life of each mutant was calculated and the mean value and the standard deviation are shown. The different X-FLAG-DTA proteins could be placed in three groups according to their *in vitro* stabilities, but since the small variations within each group (Figure 3) were not representative nor reproducible, the three groups of rapidly degraded, slowly degraded, and stable proteins have for simplicity been assigned the approximate half-lives 1 h, 4 h and >>4 h, respectively. Toxicity curves such as those shown in Figure 6A were generated for all the mutants, and the toxin concentration required to obtain 50% inhibition of protein synthesis (IC₅₀) was calculated.

X-β-gal proteins obeyed non-first-order kinetics (Baker and Varshavsky, 1991; Levy *et al.*, 1996), and the rate of degradation decreased with time. This suggested that a nascent and partially unfolded protein was more susceptible to degradation than its folded counterpart. However, in the case of the X-FLAG-DTA proteins, we could not see any substantial deviation from first-order kinetics, possibly because these proteins are mature when they encounter the degradation machinery. We therefore applied a first-order kinetic model when calculating the half-lives of the proteins, and the results are shown in Table I.

Previous work from our laboratory has shown that when the translocation of diphtheria toxin from the cell surface is induced by low pH, followed by treatment with pronase to remove extracellular material, two protected fragments are obtained, a 21 kDa polypeptide representing the A-fragment and a 25 kDa polypeptide derived from the B-fragment (Moskaug *et al.*, 1988). When the cells were subsequently permeabilized with saponin, the A-fragment was recovered from the supernatant, while the 25 kDa polypeptide remained associated with the pellet. Conceivably, some of the X-FLAG-DTA proteins might not be completely translocated to the cytosol and therefore

remain associated with membranes. Such a membrane association could modulate the stability of the protein. We therefore tested some of the X-FLAG-DTA proteins (X = Glu, Phe, Arg, Ala, Met) for their ability to be released into the supernatant upon permeabilization of the cells with saponin. We found that all these mutant A-fragments were recovered from the supernatant at a concentration of saponin where the 25 kDa fragment remained exclusively associated with the pellet (data not shown). The data indicate that all the tested mutant A-fragments are efficiently translocated to the cytosol.

***In vitro* degradation of mutant A-fragments in reticulocyte lysates**

In the case of β-galactosidase, the N-end rule has been studied both in L-cells *in vivo* (Levy *et al.*, 1996) and in reticulocyte lysates *in vitro* (Gonda *et al.*, 1989). The results obtained in these two systems with respect to the specificity of the N-end rule were similar but not identical. We also tested the stability of the various X-FLAG-DTA mutants in the reticulocyte lysate system. [³⁵S]-methionine-labelled pre-X-FLAG-DTA protein was obtained by *in vitro* translation, and X-FLAG-DTA was generated by treatment with factor Xa. Finally, an excess of fresh reticulocyte lysate was added, the sample was incubated at 37°C and aliquots were removed at intervals and analysed by SDS-PAGE and phosphorimaging (Figure 3A).

An increased duration of the treatment with factor Xa appeared to increase the *in vitro* half-life of the X-FLAG-DTA proteins (data not shown) and thus, of the different X-FLAG-DTA proteins used for the *in vitro* experiments, all were translated and treated with factor Xa in parallel. We also found that the reticulocyte lysates lost their ability to degrade the unstable X-FLAG-DTA proteins within 4 h of incubation at 37°C (data not shown), and the incubations were therefore not extended beyond 4 h. The different X-FLAG-DTA mutants could be divided into three groups with respect to their stability in the reticulocyte lysate. When X = Phe, Trp, Lys, Arg, His, a relatively rapid degradation (half-life ~1 h) of the protein was observed (Figure 3B), whereas when X = Glu, Gln, Asp, Asn, Tyr, a slow, but reproducible degradation (half-life ~4 h) could be measured (Figure 3C). In the case of the remaining proteins, we could not detect any degradation above the experimental scatter of the data (Figure 3D and data not shown). For the purpose of comparison with the *in vivo* data the approximate half-lives of X-FLAG-DTA mutants *in vitro* have been listed in Table I, and interestingly, the ten mutants that were degraded *in vitro* were also the most destabilizing ones *in vivo*.

Inhibition of in vitro degradation of X-FLAG-DTA proteins by dipeptides

Although we considered this unlikely, we could not formally exclude the possibility that the degradation of the unstable X-FLAG-DTA mutants involved a general destabilization of the protein depending on the identity of the N-terminal amino acid, rather than a process involving a specific recognition of the N-terminus. To eliminate this possibility we took advantage of the observation that short peptides mimicking the N-termini of proteins degraded by the N-end rule pathway can

Fig. 3. *In vitro* degradation of X-FLAG-DTA proteins in reticulocyte lysates. pre-X-FLAG-DTA proteins were translated *in vitro* in a reticulocyte lysate in the presence of [35 S]methionine, followed by cleavage with Factor Xa to generate the corresponding X-FLAG-DTA proteins. A sample of translation mixture was mixed with an excess of freshly thawed reticulocyte lysate, and incubated at 37°C. At the indicated time points aliquots were removed, and analysed by SDS-PAGE and phosphorimaging. The bands in the gel were quantitated, and degradation curves constructed. (A) Typical degradation patterns. (B) Rapidly degraded mutants. (C) Slowly degraded mutants. (D) Stable mutants.

inhibit the degradation of N-end rule substrates both *in vitro* (Reiss *et al.*, 1988; Gonda *et al.*, 1989) and *in vivo* (Baker and Varshavsky, 1991). Dipeptides with positively charged amino acids at the N-terminus, e.g. His-Ala, were reported to inhibit the degradation of proteins with positively charged residues at the N-terminus, while the degradation of N-end rule substrates with bulky, hydrophobic residues N-terminally could be blocked by dipeptides with similar N-termini, e.g. Trp-Ala. We failed to see any effect of the dipeptides Trp-Ala and His-Ala on the *in vivo* degradation of X-FLAG-DTA proteins in Vero-Dr22 cells (data not shown). However, so far the efficiency of such inhibitors has only been demonstrated in yeast cells (Baker and Varshavsky, 1991), and they may be inefficient in mammalian cells due to proteolysis or poor membrane permeability.

We therefore studied the effect of the dipeptides Trp-Ala and His-Ala on the degradation of X-FLAG-DTA proteins *in vitro* in reticulocyte lysates. When X = Arg, Lys, the degradation of X-FLAG-DTA *in vitro* could be efficiently inhibited by the dipeptide His-Ala, whereas the dipeptide Trp-Ala had no effect (Figure 4A-C), and similar results were obtained when X = His (data not shown). Conversely, when X = Phe, Trp, degradation was blocked by Trp-Ala, but remained unaffected by His-Ala (Figure 4A, D and E) and a similar pattern was observed when X = Tyr (data not shown).

Earlier studies have shown that a protein with N-terminal Asp, Asn, Glu or Gln is degraded by the N-end rule pathway because an Arg residue is attached to the N-terminus of the protein. In accordance with this, we found that His-Ala but not Trp-Ala, inhibited the degradation of X-FLAG-DTA when X = Glu, Gln, Asp (Figure 4F). Interestingly, a slower-migrating form of the proteins accumulated in the presence of His-Ala, while the disappearance of protein migrating at the normal rate was not affected by the dipeptide. An obvious interpretation of these data is that the slower-migrating form represents the arginylated protein, and that the conversion to the arginylated form is the rate-limiting step in the degradation process, since the slower-migrating form is not detected in the absence of His-Ala, and the disappearance of the rapidly migrating protein is not influenced by the inhibitor.

The ten amino acids that were able to destabilize X-FLAG-DTA *in vitro* were also the most destabilizing *in vivo*. One apparent discrepancy between the two systems is that while Glu and Gln were the two most destabilizing residues *in vivo*, these amino acids were only able to moderately destabilize X-FLAG-DTA *in vitro*. However, the results in Figure 4F suggest that the relatively slow degradation of the proteins with N-terminal Glu and Gln *in vitro* may simply reflect the fact that the activity involved in the conversion to the Arg-Glu form is lower in the *in vitro* system than in the living cells, and not a

Fig. 4. Inhibition of *in vitro* degradation of X-FLAG-DTA proteins by dipeptides. Experiments such as those described in Figure 3 were carried out in the presence of 10 mM of the dipeptides His-Ala or Trp-Ala as indicated. (A) Typical degradation patterns in the absence and presence of dipeptides. (B–E) Quantitation of the gel patterns shown in (A). (B) Arg-FLAG-DTA. (C) Lys-FLAG-DTA. (D) Phe-FLAG-DTA. (E) Trp-FLAG-DTA. ○, no addition; ●, His-Ala; △, Trp-Ala. (F) Effects of dipeptides on the degradation of X-FLAG-DTA proteins with Glu, Gln, or Asp at the N-terminus.

Fig. 5. Inhibition of *in vivo* degradation of X-FLAG-DTA proteins by the proteasome inhibitor lactacystin. Experiments such as those in Figure 2 were carried out, but the cells had, when indicated, been pretreated for 1 h at 37°C with 20 μ M lactacystin, which was also present during the binding of the toxin to the cells and during the incubation in growth medium at 37°C following the low pH treatment.

different specificity of the recognition component of the N-end rule pathway.

Inhibition of *in vivo* degradation of X-FLAG-DTA proteins by a proteasome inhibitor

The proteasome is a multisubunit protein complex involved in the degradation of many cellular proteins (Jentsch and Schlenker, 1995), and it has also been implicated in the degradation of N-end rule substrates in yeast (Seufert and Jentsch, 1992; Yokota *et al.*, 1996). To study whether the proteasome takes part in the *in vivo* degradation of the unstable X-FLAG-DTA proteins, we carried out experiments with two of the mutants (X = Gln, His), where the cells were treated with the proteasome inhibitor lactacystin (Fenteany *et al.*, 1995). The results (Figure 5) showed that lactacystin completely blocked the degradation of the proteins Gln-FLAG-DTA and His-FLAG-DTA. In the case of Gln-FLAG-DTA, the protein that accumulated during lactacystin treatment migrated slightly slower than the protein at $t = 0$, and a likely

explanation for this is that the conversion to the arginylated form is not affected by lactacystin. Clearly, these results indicate that the proteasome is also involved in the degradation of the N-end rule substrates in mammalian cells.

Cytotoxic effect of unstable diphtheria toxin mutants

Diphtheria toxin kills cells by catalysing the ADP-ribosylation of EF-2, leading to inhibition of protein synthesis, and it has been reported that one molecule of the A-fragment artificially introduced into the cytosol of a cell is sufficient to cause cell death (Yamaizumi *et al.*, 1978). Since one A-fragment molecule is able to catalyse the ADP-ribosylation of 2000 EF-2 molecules/min *in vitro* (Moynihan and Pappenheimer, 1981), and since one cell contains 6×10^6 EF-2 molecules (Gill and Dinius, 1973), it can be roughly estimated that one A-fragment molecule would require 30 h to inactivate all the EF-2 molecules in a cell. Therefore, if only one or a few A-fragment molecules enter the cytosol of each cell during the intoxication process, it would be expected that reducing the intracellular stability of the A-fragment would affect its cytotoxicity.

We therefore studied the capability of the various mutant toxins to inhibit protein synthesis in diphtheria toxin sensitive, wild-type Vero cells by measuring the ability of the cells to incorporate [3 H]leucine into TCA-insoluble material after overnight treatment with the toxins. The results for three of the proteins are shown in Figure 6A, and they show that the wild-type toxin, WT (half-life = 26.5 h), is more toxic than Thr-FLAG-DTA (half-life = 6.2 h), which is again more toxic than Glu-FLAG-DTA (half-life = 0.24 h). Similar experiments were performed

Fig. 6. Toxicity measurements of unstable diphtheria toxin mutants. (A) Vero cells were incubated for 24 h with increasing concentrations of reconstituted mutant toxins and the ability to incorporate [³H]leucine into TCA-precipitable material was measured. Typical curves for three of the proteins are shown. (B) The IC₅₀ value for each toxin mutant has been plotted as a function of its half-life in Vero-Dr22 cells, based on the values listed in Table I. The solid line indicates the linear regression curve calculated from the data set.

for all the mutants (data not shown) and the toxin concentration reducing the level of protein synthesis to the half-maximal value (IC₅₀) was calculated from the inhibition curves. The calculated IC₅₀ values for the different proteins are given in Table I, and in Figure 6B these values have been plotted as a function of the respective half-lives in Vero-Dr22 cells. Although the data are somewhat scattered, there is a clear correlation between the *in vivo* stability and the cytotoxicity of the proteins.

Discussion

Toxicity of unstable diphtheria toxin mutants

One of the most important features of the present study is the demonstration that a specific degradation signal can be used to reduce the toxicity of a protein toxin. Although there was an ~100-fold difference in intracellular half-life between the wild-type A-fragment and the least stable mutant, the corresponding difference in cytotoxicity was only ~20-fold. However, each mutant X-FLAG-DTA protein was generated from a corresponding precursor (pre-X-FLAG-DTA) with an N-terminal Met residue, and, at least in the case when X = Asp, the precursor was more stable and toxic than the processed protein. We consider it likely that this is also the case for the other

unstable mutants and this implies that if only a minute fraction of the precursor remained uncleaved after the factor Xa treatment, this may have considerably increased the measured toxicity of an unstable mutant. Hence, the difference in toxicity between the least and the most toxic mutants may be underestimated.

It has been claimed that when cells are intoxicated with diphtheria toxin, the A-fragment enters the cytosol as a large bolus of molecules, sufficient to inactivate rapidly all the EF-2 molecules in the cytosol (Hudson and Neville, 1985). Furthermore, it was claimed that the entry of this bolus into the cytosol, and not the rate of ADP-ribosylation of EF-2, was the rate-limiting step in the intoxication process. If this were true, one would expect that reducing the intracellular stability of the A-fragment would not affect the toxicity of the corresponding toxin. However, we found a close correlation between the intracellular stability of the mutant A-fragments and their cytotoxicity, suggesting that the ADP-ribosylation of EF-2 is actually the rate-limiting step in the intoxication process. Therefore, our data argue against the 'bolus' theory, and rather suggest that at low toxin concentrations, very few A-fragments enter the cytosol of each cell.

When mutations are introduced into the A-fragment, they may affect the cytotoxicity of the toxin through altering the catalytic activity of the A-fragment, and we cannot formally exclude that the enzymatic activity of the mutants described in this study may be reduced relative to the wild-type toxin. We think that the following points argue against any strong alterations in the enzymatic activity of the mutants used in this study: (i) the N-terminal region of the A-fragment, where the mutations were localized, is not close to the active site; (ii) if the enzymatic activity of some of the mutants was substantially altered, one would not expect such a close correlation between intracellular half-life and toxicity as that obtained; (iii) the pre-Asp-FLAG-DTA mutant, which had a much longer extension at the N-terminus than the other mutants, was one of the most toxic, and displayed a toxicity close to that of the wild-type toxin (only a 2-fold reduction).

Comparison with previous studies on the specificity of the N-end rule

Three systematic studies on the specificity of the N-end rule in eukaryotic cells have previously been published. In Figure 7A, we have plotted the *in vivo* half-lives of the X-FLAG-DTA proteins studied in the present work according to increasing stability, and in Figure 7B are presented the half-lives of different X-β-gal proteins in yeast (Bachmair *et al.*, 1986), using the same order of amino acid residues. The specificity of the N-end rule obtained for X-FLAG-DTA in Vero-Dr22 cells is quite similar to that obtained with X-β-gal proteins in yeast, the most striking difference being that Leu and Ile are destabilizing in yeast but not in Vero-Dr22 cells. Also, in yeast the amino acids could easily be separated into two groups with respect to stability, while the X-FLAG-DTA proteins displayed a continuum of stabilities, making it more difficult to draw any border between stabilizing and destabilizing amino acids.

The same X-β-gal proteins used for the study in yeast have also been tested *in vitro* in reticulocyte lysates (Gonda *et al.*, 1989), and the results obtained (Figure 7C) were

Fig. 7. Comparison of the specificity of the N-end rule between different systems. **(A)** The *in vivo* half-lives of different X-FLAG-DTA proteins have been plotted as a function of the N-terminal amino acid X according to increasing stability. **(B)** Plot of the stability of X-β-gal proteins in yeast (Bachmair *et al.*, 1986) maintaining the order of amino acids used in (A). **(C)** Similar plot of half-lives of X-β-gal proteins in reticulocyte lysates (Gonda *et al.*, 1989). **(D)** *In vivo* stabilities of X-β-gal proteins in L-cells calculated from Levy *et al.*, (1996). The degradation in this system did not follow first-order kinetics, and the degradation rate decreased with time. Thus, the stabilities of the proteins were expressed as initial decay (ID¹⁰) which represents the degradation of X-β-gal relative to Met-β-gal during a 10 min pulse of labelling with [³⁵S]methionine. In the figure remaining protein (100-ID¹⁰) is plotted as a function of the N-terminal residue, using the same order of amino acids as in (A).

quite similar to those obtained with X-FLAG-DTA in Vero-Dr22 cells and in reticulocyte lysates. The major difference was that Cys was strongly destabilizing in the case of X-β-gal, but not in the case of X-FLAG-DTA. The stability of X-β-gal proteins has also been studied *in vivo* in L-cells (Levy *et al.*, 1996; Figure 7D), and again the primary discrepancy with our results is that Cys is destabilizing for X-β-gal in L-cells, but not for X-FLAG-DTA in Vero-Dr22 cells. We have tested some of the X-FLAG-DTA proteins (X = Phe, Arg, Glu, Cys, Met) in CHO cells transfected with the diphtheria toxin receptor, and also in this hamster cell line we found that Glu, Phe and Arg were destabilizing while Met and Cys were stabilizing (our unpublished data). It therefore appears that the stability of Cys-FLAG-DTA in Vero cells does not represent a special feature of this cell line, e.g. a defect in the enzyme(s) adding Arg to N-terminal Cys residues.

Altogether, the results from the present study confirm the main features of the N-end rule as previously published. However, the discrepancy obtained in the case of Cys suggests that the specificity of the N-end rule may not be

completely invariant between different substrate proteins. Since Cys-FLAG-DTA was stable both in reticulocyte lysates and in Vero and CHO cells, while Cys-β-gal was unstable both in reticulocyte lysates and L cells, one may speculate that the context of the N-terminal amino acid residue may influence how it is recognized by the N-end rule pathway.

We did not find any appreciable differences between the specificity on the N-end rule obtained with X-FLAG-DTA proteins *in vivo* in Vero-Dr22 cells and that obtained *in vitro* in reticulocyte lysates. However, there are some differences between the results obtained with X-β-gal proteins *in vitro* in reticulocyte lysates (Gonda *et al.*, 1989; Figure 7C) and those obtained in L-cells (Levy *et al.*, 1996; Figure 7D). In particular, Thr, Ser and Ala were able to destabilize X-β-gal *in vitro*, but not *in vivo*. However, the X-β-gal proteins used in the two different studies had different sequences in the N-terminal region, and our results suggest that this might be the reason for the discrepancies between the two systems, rather than a difference in the N-end rule between reticulocytes and nucleated cells.

N-terminal amino acids of toxins that reach the cytosol

Many bacterial and plant toxins act on intracellular targets, and since the part of the toxin that enters the cytosol is usually generated from a precursor, this protein can in theory have any amino acid at the N-terminus. If a destabilizing amino acid at the N-terminus of a toxin were sufficient to make the protein unstable in the cytosol, one would expect that throughout evolution there would have been a selection against destabilizing amino acids at the N-termini of toxic proteins that reach the cytosol. Therefore, we investigated the published N-termini of some of the toxin domains that are believed to enter the cytosol (DeLange *et al.*, 1979; Lamb *et al.*, 1985; Nicosia *et al.*, 1986; Calderwood *et al.*, 1987; Yamamoto *et al.*, 1987; Escuyer *et al.*, 1988; Strockbine *et al.*, 1988; Bragg and Robertson, 1989; Ogata *et al.*, 1990; Krieglstein *et al.*, 1991; Wood *et al.*, 1991; Kurazono *et al.*, 1992; Huguet Soler *et al.*, 1996), but we could not find any systematic exclusion of destabilizing residues at the N-termini of the toxin domains. Although it cannot be formally excluded that minor modifications of the N-termini of these proteins occur upon translocation, the data suggest that placing a destabilizing residue at the N-terminus may often not be sufficient to make a protein unstable. This is supported by the observations that RNase A, which has an N-terminal Lys residue, is very stable *in vivo* (Rote and Rechsteiner, 1986), and that placing a destabilizing residue at the N-terminus of dihydrofolate reductase is insufficient for targeting this protein for degradation by the N-end rule pathway (Bachmair and Varshavsky, 1989). When the N-end rule was initially discovered in yeast, it was also found that amino acids that are destabilizing according to the N-end rule are quite rare in cytosolic proteins and quite common in compartmentalized and secreted proteins. It may therefore seem paradoxical that destabilizing amino acids are allowed in toxic proteins entering from the outside, but not in cytosolic proteins expressed within the cell. However, one must bear in mind that the repertoire of amino acids at the N-termini of cytosolic proteins is governed by the specificity of the methionine aminopeptidase, which removes the initiator methionine from a protein only if the amino acid in the second position belongs to a subset of small amino acids, i.e. Gly, Ala, Ser, Thr, Pro, Val, Cys (Huang *et al.*, 1987; Arfin and Bradshaw, 1988; Boissel *et al.*, 1988) that are usually stabilizing according to the N-end rule.

Potential use of unstable toxins

To our knowledge, the present study is the first to report that the life-span and toxicity of a toxic protein that enters the cell from the exterior can be modulated by adding a degron. Other studies have also reported mutants of diphtheria toxin with reduced intracellular stability (Yamaizumi *et al.*, 1982; Falnes *et al.*, 1995), but in these cases the shorter half-life is likely to be caused by a general destabilization of the protein due to abolished NAD binding (Lory *et al.*, 1980; Yamaizumi *et al.*, 1982) or an increased hydrophobicity (Falnes *et al.*, 1995), and not a specific recognition of a degron.

The observation that a toxin can be destabilized by a degron opens up interesting possibilities. Varshavsky has proposed that toxins containing several degrons that can

be masked by specific proteins, will be active only in cells that express all of these proteins in the cytoplasm, and that such toxins may represent a new type of drug with higher specificity than conventional drugs (Varshavsky, 1995b). Indeed, we have preliminary data indicating that the degradation of Asp-FLAG-DTA *in vitro* is inhibited by the anti-FLAG M1 antibody which specifically recognizes the FLAG epitope when localized N-terminally in a protein (our unpublished observations). However, this antibody only recognizes the epitope in the presence of a high concentration of calcium and would thus not be suitable for inhibiting the degradation of Asp-FLAG-DTA in the cytosol. On the other hand, the FLAG M2 antibody recognizes the FLAG peptide independently of calcium, and also, the first Asp residue appears to be non-critical for recognition (Miceli *et al.*, 1994). This antibody may thus be suitable for inhibiting the degradation of many different X-FLAG-DTA proteins *in vivo*.

The diphtheria toxin A-fragment inhibits cellular protein synthesis through catalysing the ADP-ribosylation of EF-2, thereby inactivating this crucial component of the translation machinery. Since one molecule of wild-type diphtheria toxin is sufficient to kill a cell, it has previously not been possible to investigate whether the cell has some way of reversing the modification of EF-2. However, by using an unstable mutant of the A-fragment that will be active in the cytosol only for a limited time period and then measuring cellular protein synthesis as a function of the time after the A-fragment was introduced into the cytosol, one may be able to address this question.

Materials and methods

Buffers, media and reagents

Dialysis buffer contained 140 mM NaCl, 20 mM HEPES and 2 mM CaCl₂, adjusted to pH 7.0 with NaOH. HEPES medium contained bicarbonate- and serum-free Eagle's minimal essential medium buffered with HEPES to pH 7.4. Lysis buffer contained 0.1 M NaCl, 20 mM NaH₂PO₄, 10 mM EDTA, 1% Triton X-100, 1 mM PMSF and 1 mM NEM, pH 7.4. MES-gluconate buffer contained 140 mM NaCl, 5 mM Na-gluconate and 20 mM MES, adjusted with Tris to pH 4.8. PBS was 140 mM NaCl and 10 mM NaH₂PO₄, pH 7.4. Lactacystin was obtained from E.J. Corey.

Cell cultures

Mutant and wild-type Vero cells were grown in Dulbecco's Modified Eagle's Medium in the presence of 5% fetal calf serum. For degradation experiments, the cells were seeded into 12-well Costar (Cambridge, MA) microtitre plates at a density of 2×10^5 cells/well on the day preceding the experiments, and for toxicity experiments the cells were seeded out in 24-well plates at a density of 5×10^4 cells/well.

Construction of mutant plasmids

PCR was used to construct the plasmids encoding the different pre-X-FLAG-DTA proteins, and the plasmid pKD-52 (Ariansen *et al.*, 1993), encoding the wild-type toxin, was used as template. As forward mutant primers we used CAGTGCCATGGCACATATCGAGGGAAGGNNNTACAAGGACGACGATGACAAGCTCATGGCTGATGATGTTGTGAT, where the underlined sequence anneals to the sequence corresponding to codons 1 to 7 in the toxin gene, and the triplet NNN represents partial or complete wobbling (to make up the majority of the mutants) and in some cases specific codons (to construct the remaining ones). As a reverse primer we used CCTGCACAGGCTTGAGCC (annealing to the sequence encompassing codons 183–188 in the toxin gene). The resulting PCR product was digested with *Nco*I and *Ce*III, and cloned between the *Nco*I and *Ce*III sites of pKD-52. The sequences that had been amplified by PCR were verified by dideoxy sequencing. The plasmid pKD-66, encoding pre-Phe-DTA, is also based on pKD-52 and was constructed by standard cloning techniques.

***In vitro* transcription and translation**

Plasmid DNA was linearized downstream of the encoding gene and transcription was carried out in a 20 µl reaction mixture with T3 RNA polymerase as described (McGill *et al.*, 1989). The mRNA was precipitated with ethanol and dissolved in 10 µl H₂O containing 10 mM DTT and 0.1 U/µl RNasin (Promega, Madison, WI). The translation was performed for 1 h at 30°C in micrococcal nuclease-treated rabbit reticulocyte lysate (Promega, Madison, WI) containing 1 µM [³⁵S]methionine and 25 µM of each of the 19 other amino acids, and 5 µl of the dissolved mRNA was used per 100 µl of lysate. When unlabelled B-fragment was prepared, labelled methionine was replaced by 20 µmol unlabelled methionine.

***In vitro* reconstitution of active toxin**

When A- and B-fragments are mixed under reducing conditions and the reducing agent is subsequently removed by dialysis, active heterodimeric toxin will form quantitatively (Stenmark *et al.*, 1992). A- and B-fragments were made separately by *in vitro* transcription and translation, the translation mixtures were mixed together in a 1:1 ratio and formation of the interfragment disulfide bond was facilitated by overnight dialysis against dialysis buffer. In the cases where the toxin was generated by cleavage of a precursor with factor Xa, the reconstituted toxin was treated with 1 µg factor Xa per 100 µl of reticulocyte lysate for 1 h at 37°C.

SDS-PAGE

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was carried out in 12% gels as described by Laemmli (1970). After electrophoresis, the gel was fixed for 30 min in 27% methanol/4% acetic acid, and then incubated for 30 min in 1 M Na-salicylate/2% glycerol, pH 5.8. Kodak XAR-5 film was exposed to the dried gel in the absence of intensifying screen at -80°C. In some cases phosphor storage screens (Molecular Dynamics, Sunnyvale, CA) were exposed to the gels.

Assay for analysing *in vivo* degradation of mutant A-fragments

Vero-Dr22 cells were incubated for 20 min at 25°C with mutant or wild-type diphtheria toxin (1 nM), reconstituted from *in vitro*-expressed [³⁵S]methionine-labelled A-fragment and unlabelled B-fragment. The cells were washed three times with PBS to remove unbound toxin, and subsequently incubated for 3 min at 37°C with MES-gluconate buffer, pH 4.8, to induce the translocation of the A-fragment to the cytosol. The cells were subsequently incubated at 37°C in cell growth medium for various time periods. Finally, the cells were lysed for 10 min at 0°C in lysis buffer, cellular proteins were collected by TCA precipitation and analysed by non-reducing SDS-PAGE and fluorography. In experiments where the radioactive labelling of the protein was relatively weak, the bands in the gels were quantitated by densitometric scanning of the films. In the other cases, phosphor storage screens were exposed to the gels, and the gel image was analysed in a Molecular Dynamics Phosphorimager (Sunnyvale, CA).

Assay for analysing degradation of mutant A-fragments *in vitro* in a reticulocyte lysate

[³⁵S]methionine-labelled A-fragments obtained by *in vitro* translation were cleaved with 1 µg factor Xa per 100 µl of translation mixture for 1 h at 37°C. To 4 µl of [³⁵S]methionine-labelled A-fragment was added 16 µl of fresh reticulocyte lysate, and the mixture was incubated at 37°C. In the experiments where the inhibitory effect of dipeptides was studied, bestatin (40 µg/ml) was also present, since this compound has been reported to potentiate the effect of such inhibitors (Reiss *et al.*, 1988). At different time points, 3 µl aliquots were removed, added to 27 µl of reducing SDS-PAGE sample buffer and immediately frozen. Subsequently, the samples were analysed by SDS-PAGE and phosphorimaging. To prevent overloading of the gel only one-third of each sample was applied to the gel.

Measurement of toxicity of mutant toxins

Vero cells were incubated for 24 h at 37°C with increasing concentration of wild-type and mutant toxins. The cells were allowed to incorporate radioactivity for 30 min at 37°C in HEPES medium containing 2 µCi/ml [³H]leucine and no unlabelled leucine. The cells were washed for 10 min with 5% TCA, followed by another brief wash with 5% TCA, finally dissolved in 0.1 M KOH and the cell-associated radioactivity was measured. The amount of reconstituted toxin in the reticulocyte lysates used in the toxicity experiments was estimated by quantitative Western blotting.

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