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New Concepts in Biochemistry

Accumulating Evidence Suggests That Several AB-Toxins Subvert the Endoplasmic Reticulum-Associated Protein Degradation Pathway To Enter Target Cells†

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ABSTRACT: Several AB-toxins appear to have independently evolved mechanisms by which they undergo retrograde transport from the cell membrane to the endoplasmic reticulum (ER). Recent insights into ER-associated protein degradation (ERAD) now provide clues as to why these toxins have selected the ER as the site of cell entry. We propose that they disguise themselves as misfolded proteins to enter the ERAD pathway. We further link the observation that these toxins have few, if any, lysine residues to the need to escape ubiquitin-mediated protein degradation, the ultimate destination of the ERAD pathway. The actual membrane translocation step remains unclear, but studies on viral immune evasion mechanisms indicate that retrotranslocation across the ER lipid bilayer may involve SEC61. Understanding the internalization process of these toxins opens new avenues for preventing their entry into cells. In addition, this knowledge can be exploited to create protein-based pharmaceuticals that act on cytosolic targets.

Plants and bacteria produce a number of protein toxins that act on cytosolic targets in mammalian cells. To reach their targets, these toxins must therefore cross the lipid bilayer. In one group of toxins, referred to as the AB-toxins, the task of catalyzing the toxic reaction is structurally separated from the task of targeting the toxic activity. The two structural components that carry out these tasks are referred to as the catalytically active A component and the cell binding B component (*1*). The mechanisms by which the AB-toxins reach the cytosol are now starting to unfold. Basically, the entry process is initiated by binding to cell-surface receptors, followed by endocytosis and vesicular trafficking to the site of membrane translocation. For one subset of AB-toxins, exemplified by diphtheria toxin (DT), the site of translocation is the acidic endolysosomal compartment. Here, membrane translocation is initiated by a pH-induced conformational rearrangement (*2*). As a consequence, these toxins are inhibited by agents that prevent acidification of the lysosome (*2*). In this paper, we focus on another group of AB-toxins whose toxicity is blocked by Brefeldin A (*3*−*8*), a drug that disrupts the Golgi apparatus. Well-known members of this group are cholera toxin (CT), heat-labile enterotoxin (LT), pertussis toxin (PT), *Pseudomonas aeruginosa* exotoxin A (ExoA), Shiga toxin (ST), Shiga-like toxins (SLTs), and ricin. For several members of this group, it is now clear that the function of the Golgi is to act as a gateway through which the toxin can reach the endoplasmic reticulum (ER).

Intracellular Trafficking to the ER

As was outlined above, cell entry of AB-toxins starts by binding of the B component to cell-surface receptors, often glycolipids or glycoproteins. After endocytic uptake, the ER-directed toxins are then transported to the trans Golgi network where they enter the Golgi apparatus (*9*, *10*). The next journey takes the toxin back to the ER. CT, LT, and ExoA may achieve this by means of their C-terminal ER-retrieval

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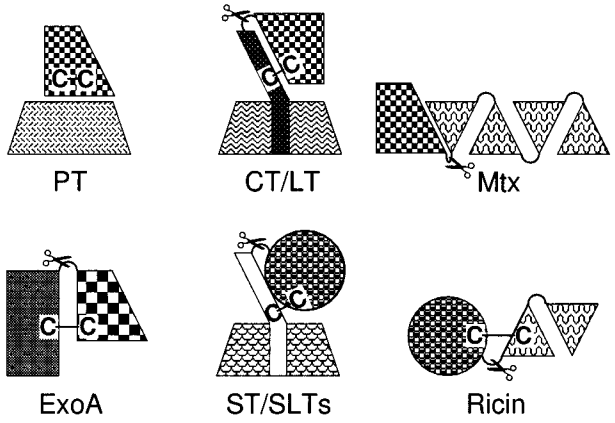

FIGURE 1: The ER-directed AB-toxins can be divided into six distinct classes. In this figure, identical shapes indicate conserved structure whereas identical fill patterns indicate conserved sequence. The pair of scissors indicates a proteolysis-sensitive loop which is cleaved in a pre-Golgi compartment (*24*, *44*). Disulfide bonds are represented by C−C and are reduced in a post-Golgi compartment (*24*, *25*). Mtx is a 100 kDa mosquitocidal toxin of *Bacillus sphaericus* (*46*). The effect of Brefeldin A on Mtx toxicity has not been determined, but the low lysine frequency in its A component (see Figure 3) suggests that it is a true ER-directed AB-toxin. The A component of ExoA is only very distantly related to PT, CT/LT, and Mtx; this has been indicated by using larger squares in its fill pattern.

signal, a KDEL or related tetrapeptide. Soluble proteins with an ER-retrieval signal are transported back from the Golgi to the ER by the KDEL receptor (*11*, *12*), and retrieval from compartments as far downstream as the trans Golgi network has been reported (*13*). Mutagenesis studies on ExoA indeed show that its ER-retrieval sequence is required for toxicity (*14)*. For CT and LT, the ER-retrieval sequence appears not to be required absolutely (*15*, *16*); however, it enhances the efficient delivery of the toxin (*16*). For the toxins that lack an ER-retrieval signal, it is not clear how they reach the ER. However, Shiga toxin has been visualized in the ER using electron microscopy (*17*), and the transport of ricin to the ER has been established (*18*). The most detailed information is currently available for CT (*19*−*22*), and it appears that its A and B components dissociate in the Golgi (*19, 21*). However, for the other toxins, it seems likely that dissociation takes place in the ER itself (*17*, *23*, *24*). In many cases, dissociation may result from the cleavage of a disulfide bond that links the A and B components (Figure 1), possibly catalyzed by an ER-resident protein disulfide bond isomerase (*25*). For PT, we have proposed that dissociation is induced by ATP binding in the ER (*23*).

Why the ER?

At first, it may seem surprising that toxins travel by retrograde transport all the way to the ER. An attractive explanation would be that the barrier that separates the cytosol from the extracellular space can be breached easily from the ER (*26*). This explanation is strengthened by the observation that the trafficking to the ER has been developed independently in at least six different toxins (Figure 1). Two recent discoveries now present direct evidence that proteins can indeed return from the ER to the cytosol (retrotranslocation). Retrotranslocation may even constitute part of a normal cellular process known as ER-associated degradation (ERAD) (*27*).

CT	**YGLAGFPPEHRAWREEPWIHHAPPGCG**NAPRSS▼MS
LT-I	**YRLAGFPPDHQAWREEPWIHHAPQGCG**DSSR▼TITG
LT-IIA	**YQLAGFPSNFPAWREMPWSTFAPEQCV**PNNK▼EFKG
PT	**VASIVGTLVRMAPVIGACMA**
ricin	**FSVYDVSILIPIIALMVYRCAPPP**SSQF▼SL
ST/SLT-I	**ISFGSINAILGSVALILNCHHHASRVAR**▼MA
SLT-II	**ISFNNISAILGTVAVILNCHHQGARSVR**▼AV
ExoA	PR▼GWEQLEQCGYPVQRLVALYLAARLSW

FIGURE 2: Hydrophobic peptides that become exposed upon dissociation of the A and B components. The hydrophobic segment is printed in bold font. LT-I and LT-IIA as well as SLT-I and SLT-II are different toxin variants. Note that all peptides contain a cysteine residue, underlined, that is believed to be reduced in the ER. All toxins, except PT, also have an adjacent site, indicated by a filled triangle, which is proteolytically cleaved prior to arrival in the ER. Cleavage of these two covalent bonds and the dissociation of the toxins are likely to destabilize the structure of the hydrophobic peptides. The hydrophobic peptides of PT, ST, SLT, and ricin have previously been reported to be potential membrane-spanning helices (*47*, *48*), and membrane insertion of the ST peptide has been demonstrated (*48*). Our hypothesis suggests an alternative role for these peptides, but this does not necessarily exclude their previously proposed function.

ER-Associated Degradation

For many years, it has been known that some proteolytic machinery associated with the ER removes proteins that fail to fold properly (*28*). However, only in the last two years has it become clear that the actual proteolysis is carried out by the proteasome in the cytosol (*27*). Accordingly, there must be a mechanism that targets misfolded proteins back to the cytosol. For the cystic fibrosis transmembrane conductance regulator (CFTR), an integral membrane protein, it has been shown that ubiquitination of the cytosolic domain is involved in degradation (*29*). For some lumenal ER proteins, calnexin may be involved, since degradation of pro-α factor was inhibited in a yeast strain with a disrupted calnexin gene (*30*). In addition, ubiquitination of the cytosolic domain of calnexin was found to be induced by the expression of a lumenal ER protein with a folding defect (*31*). Lumenal ER proteins can also become ubiquitinated themselves, after retrotranslocation to the cytosol, as was shown for carboxypeptidase yscY (*32*), but ubiquitin-independent degradation has also been reported (*33*). It is unknown how proteins with a folding defect enter the ERAD pathway, but calnexin is an ER-resident chaperone and would therefore be well-suited to interacting with improperly folded proteins. It remains to be established how such a pathway can distinguish proteins that are intermediates in a normal folding process from proteins that cannot fold properly. Brodsky and McCracken (*27*) speculate that the lifetime of the complexes may be the deciding factor.

Virus-Induced Retrotranslocation

Studies on the suppression of major histocompatibility complex class I (MHC-I) expression by viruses has revealed a novel strategy in the human cytomegalovirus (HCMV). This virus produces two proteins, US2 and US11, that have a leader sequence and are consequently exported cotranslationally into the ER. After arrival in the ER, US2 and US11 cause newly produced MHC-I chains to be returned to the cytosol where they are degraded by the proteasome (*34*, *35*). The details of the mechanism remain to be worked out, but immunoprecipitation studies showed that the retrotranslocated MHC-I molecules are closely associated with SEC61. This suggests that the return to the cytosol involves the same

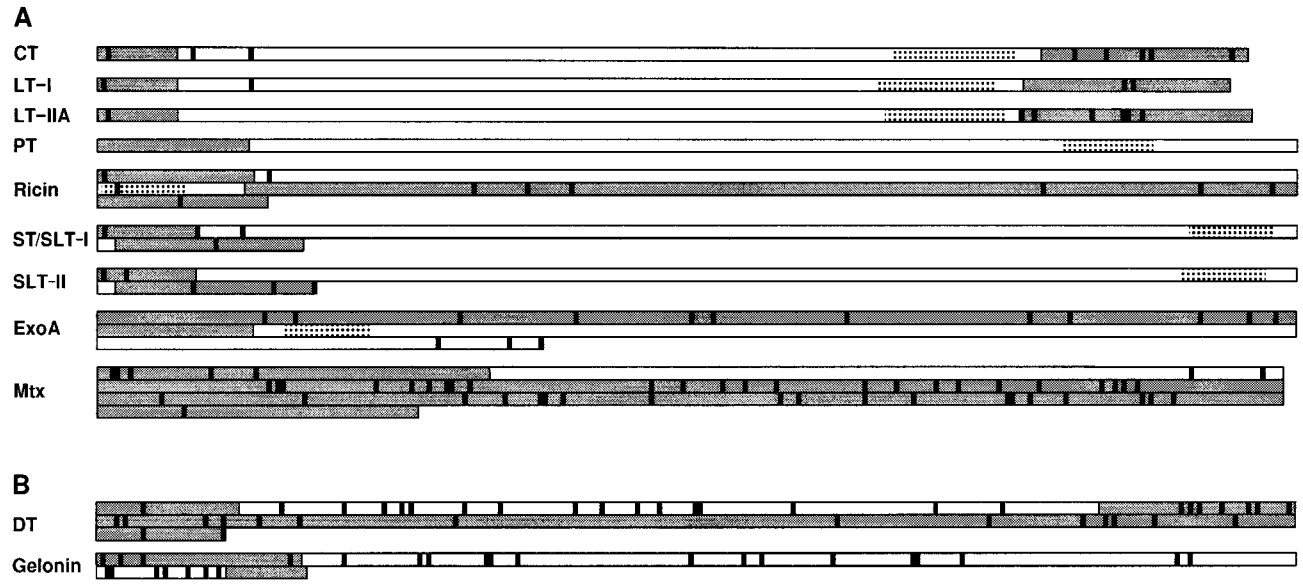

FIGURE 3: (A) Schematic sequence representation of ER-directed AB-toxins. Gray areas are removed before the A component, blank area, reaches the cytosol. The full sequences, including leader sequences, are shown. Dotted areas in the A components represent the hydrophobic sequences listed in Figure 2. Vertical black bars represent lysine residues. Note that lysine residues occur only near the N or C termini of the A component. In contrast, lysine residues have a normal distribution outside the A component. (B) Schematic sequence representation as in panel A, but now for two toxins that do not enter the cytosol via the ER. Note that lysine residues are distributed throughout the sequences.

protein translocation pore that is used in the normal anterograde direction (*34*). It will be interesting to see if US2 and US11 create a new retrotranslocation pathway or whether they exploit a normal host pathway, such as the ERAD pathway. The observation that misfolding of MHC-I, in the absence of US2 or US11, also leads to an increased association with SEC61 provides the first insight into the possibility that the two retrotranslocation pathways may indeed be connected (*34*).

AB-Toxin Retrotranslocation

At this moment, there are no experimental data to support the mechanism of membrane translocation for the ER-directed AB-toxins. However, the knowledge that proteins can be returned from the ER to the cytosol suggests that AB-toxins developed the complex routing to the ER in order to exploit this property. To do so, the toxins have to enter the ERAD pathway, but at the same time, they must escape its ultimate fate, degradation. Rapak *et al.* (*18*) independently proposed a link between the cell entry of AB-toxins and the ERAD pathway, but they did not address how the toxins entered the pathway or how they escaped degradation. We propose that the toxins enter the pathway by disguising themselves as proteins with a folding defect. Inspection of the structures of the ER-directed AB-toxins suggests that they can accomplish this by exposing hydrophobic regions upon dissociation of their A and B components in the ER. For instance, CT and LT have a small hydrophobic subdomain, the $A1_3$ domain (*36*), that is tethered to the compact catalytic domain via a flexible 29-residue linker. This domain is stabilized by packing interactions in the holotoxin and by a disulfide bond. Disulfide bond reduction and dissociation of the holotoxin may well leave the hydrophobic $A1_3$ domain in an unfolded state. Similar hydrophobic peptides are present in the other toxins (Figure 2), although for ST it is less likely that this peptide can be easily unfolded. A final observation of interest is that for CT, PT, and ricin it is known that the A component interacts more strongly with lipid membranes after dissociation and disulfide reduction (*37*−*39*). This may also indicate that hydrophobic regions become exposed upon holotoxin dissociation. In summary, we propose that the ER-directed AB-toxins expose a hydrophobic and possibly unfolded peptide upon dissociation of their A and B components. This would mark the toxins for retrotranslocation by the ERAD pathway. The main problem with entry into the ERAD pathway is that it leads to protein degradation. However, a sequence analysis of AB-toxins shows that they have an elegant built-in mechanism that could exactly avoid this fate.

Low Lysine Content in AB-Toxins

When the sequences of ER-directed AB-toxins became available, it was noted that lysine residues were very uncommon, if not absent, in their A components (*40*). Interestingly, their B components and the AB-toxins that enter the cytosol from the lysosome have a normal lysine content (Figure 3). It should be further noted that the arginine content is normal or often even elevated (not shown). Several reasons for this sequence bias have been put forward, but none were satisfactory (*40*). We have proposed that the lack of lysine residues was a mechanism for escaping ubiquitin-mediated protein degradation during translocation of ER-directed AB-toxins into the cytosol (*41*). We were led to this proposal by the knowledge that ubiquitin attachment requires lysine residues. D. J. FitzGerald has also inferred a possible connection to ubiquitination (personal communication). Further support for this idea, and a rationale for why only ER-directed AB-toxins are affected, came from work that showed that there is a specific ubiquitin-conjugating enzyme, UBC6, attached to the cytosolic side of the ER membrane (*42*). Moreover, a yeast strain with a temperature-sensitive defect in SEC61 was rescued in a UBC6 knockout. This suggests that (partly) unfolded proteins near the ER membrane are a target for UBC6-

catalyzed ubiquitination (*42*). UBC6 has indeed been implicated in degradation of proteins via the ERAD pathway (*32*), and MHC-I degradation in a compartment associated with ubiquitin-conjugating enzymes has been reported (*43*).

Upon closer inspection of the amino acid sequences of the catalytic domains of ER-directed AB-toxins, one notes that if lysine residues are present they are near either the N or C terminus [see Figure 3 and the work of London and Luongo (*40*)]. This apparently reduced evolutionary pressure against lysine residues near the termini of the polypeptide chain suggests that ubiquitination is less likely at these positions. Furthermore, the complete lack of internal lysine residues suggests that all of these positions are potential targets for ubiquitination, which implies that the toxins are unfolded during translocation.

Conclusion

Pathogens are known to be masters of exploiting normal physiologic processes for their own benefit. In this paper, we argue that a group of AB-toxins have evolved mechanisms to travel from the cell membrane to the ER in order to exploit the retrotranslocation pathway that forms part of the ER-associated degradation process. Even during their journey to the ER, they exploit processes like ER retrieval by the KDEL receptor, activation by host proteases (*44*) and protein disulfide bond isomerases, and binding-induced endocytic uptake. In turn, several laboratories are now copying the ability of AB-toxins to deliver toxins to specific cell types by coupling a toxin to a tumor-directed targeting component (*45*). Knowing how the AB-toxins exploit and avoid cellular processes will be of great importance for the effective design of this new class of pharmaceuticals.

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REFERENCES

1. Gill, D. M. (1978) in *Bacterial toxins and cell membranes* (Jeljaszewica, J., and Wadstrom, T., Eds.) pp 291−332, Academic Press, New York.
2. Draper, R. K., and Simon, M. I. (1980) *J. Cell Biol. 87*, 849−854.
3. Yoshida, T., Chen, C., Zhang, M., and Wu, H. C. (1991) *Exp. Cell Res. 192*, 389−395.
4. Nambiar, M. P., Oda, T., Chen, C., Kuwazuru, Y., and Wu, H. C. (1993) *J. Cell Physiol. 154*, 222−228.
5. Orlandi, P. A., Curran, P. K., and Fishman, P. H. (1993) *J. Biol. Chem. 268*, 12010−12016.
6. Donta, S. T., Beristain, S., and Tomicic, T. K. (1993) *Infect. Immun. 61*, 3282−3286.
7. Xu, Y., and Barbieri, J. T. (1995) *Infect. Immun. 63*, 825−832.
8. Donta, S. T., Tomicic, T. K., and Donohue-Rolfe, A. (1995) *J. Infect. Dis. 171*, 721−724.
9. Joseph, K. C., Kim, S. U., Stieber, A., and Gonatas, N. K. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2815−2819.
10. Lendaro, E., Ippoliti, R., Bellelli, A., Brunori, M., Evangelista, V., Guidarini, D., and Benedetti, P. A. (1994) *FEBS Lett. 344*, 99−104.
11. Pelham, H. R. B. (1990) *Trends Biochem. Sci. 15*, 483−486.
12. Nilsson, T., and Warren, G. (1994) *Curr. Opin. Cell Biol. 6*, 517−521.
13. Miesenbock, G., and Rothman, J. E. (1995) *J. Cell Biol. 129*, 309−319.
14. Chaudhary, V. K., Jinno, Y., FitzGerald, D. J., and Pastan, I. (1990) *Proc. Natl. Acad. Sci. U.S.A.* 87, 308−312.
15. Cieplak, W., Jr., Messer, R. J., Konkel, M. E., and Grant, C. C. R. (1995) *Mol. Microbiol. 16*, 789−800.
16. Lencer, W. I., Constable, C., Moe, S., Jobling, M. G., Webb, H. M., Ruston, S., Madara, J. L., Hirst, T. R., and Holmes, R. K. (1995) *J. Cell Biol. 131*, 951−962.
17. Sandvig, K., Ryd, M., Garred, Ø., Schweda, E., Holm, P. K., and van Deurs, B. (1994) *J. Cell Biol. 126*, 53−64.
18. Rapak, A., Falnes, P. Ø., and Olsnes, S. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 3783−3788.
19. Majoul, I. V., Bastiaens, P. I. H., and Söling, H.-D. (1996) *J. Cell Biol. 133*, 777−789.
20. Sofer, A., and Futerman, A. H. (1996) *J. Neurochem. 67*, 2134−2140.
21. Bastiaens, P. I. H., Majoul, I. V., Verveer, P. J., Söling, H.-D., and Jovin, T. M. (1996) *EMBO J. 15*, 4246−4253.
22. Sandvig, K., Garred, Ø., and van Deurs, B. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 12339−12343.
23. Hazes, B., Boodhoo, A., Cockle, S. A., and Read, R. J. (1996) *J. Mol. Biol. 258*, 661−671.
24. Ogata, M., Chaudhary, V. K., Pastan, I., and FitzGerald, D. J. (1990) *J. Biol. Chem. 265*, 20678−20685.
25. Majoul, I., Ferrari, D., and Söling, H.-D. (1997) *FEBS Lett. 401*, 104−108.
26. Pelham, H. R. B., Roberts, L. M., and Lord, J. M. (1992) *Trends Cell Biol. 2*, 183−185.
27. Brodsky, J. L., and McCracken, A. A. (1997) *Trends Cell Biol. 7*, 151−156.
28. Klausner, R. D., and Sitia, R. (1990) *Cell 62*, 611−614.
29. Ward, C. L., Omura, S., and Kopito, R. R. (1995) *Cell 83*, 121−127.
30. McCracken, A. A., and Brodsky, J. L. (1996) *J. Cell Biol. 132*, 291−298.
31. Qu, D., Teckman, J. H., Omura, S., and Perlmutter, D. H. (1996) *J. Biol. Chem. 271*, 22791−22795.
32. Hiller, M. M., Finger, A., Schweiger, M., and Wolf, D. H. (1996) *Science 273*, 1725−1728.
33. Werner, E. D., Brodsky, J. L., and McCracken, A. A. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 13797−13801.
34. Wiertz, E. J. H. J., Tortorella, D., Bogyo, M., Yu, J., Mothes, W., Jones, T. R., Rapoport, T. A., and Ploegh, H. L. (1996) *Nature 384*, 432−438.
35. Wiertz, E. J. H. J., Jones, T. R., Sun, L., Geuze, H. J., and Ploegh, H. L. (1996) *Cell 84*, 769−779.
36. Zhang, R.-G., Scott, D. L., Westbrook, M. L., Nance, S., Spangler, B. D., Shipley, G. G., and Westbrook, E. M. (1995) *J. Mol. Biol. 251*, 563−573.
37. Ishida, B., Cawley, D. B., Reue, K., and Wisnieski, B. J. (1983) *J. Biol. Chem. 258*, 5933−5937.
38. Hausman, S. Z., and Burns, D. L. (1992) *J. Biol. Chem. 267*, 13735−13739.
39. Tomasi, M., and Montecucco, C. (1981) *J. Biol. Chem. 256*, 11177−11181.
40. London, E., and Luongo, C. L. (1989) *Biochem. Biophys. Res. Commun. 160*, 333−339.
41. Stein, P. E., Hazes, B., and Read, R. J. (1996) in *Protein toxin structure* (Parker, M. W., Ed.) pp 191−216, R. G. Landes Co., Austin, TX.
42. Sommer, T., and Jentsch, S. (1993) *Nature 365*, 176−179.
43. Raposo, G., van Santen, H. M., Leijendekker, R., Geuze, H. J., and Ploegh, H. L. (1995) *J. Cell Biol. 131*, 1403−1419.
44. Gordon, V. M., and Leppla, S. H. (1994) *Infect. Immun. 62*, 333−340.
45. Ghetie, M. A., and Vitetta, E. S. (1994) *Curr. Opin. Immunol. 6*, 707−714.
46. Thanabalu, T., Hindley, J., Jackson-Yap, J., and Berry, C. (1991) *J. Bacteriol. 173*, 2776−2785.
47. Stein, P. E., Boodhoo, A., Armstrong, G. D., Cockle, S. A., Klein, M. H., and Read, R. J. (1994) *Structure 2*, 45−57.
48. Saleh, M. T., Ferguson, J., Boggs, J. M., and Gariépy, J. (1996) *Biochemistry 35*, 9325−9334.

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