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PERTACTIN PROCESSING AND SECRETION IN PROTEASE(S) DEFICIENT STRAINS OF *ESCHERICHIA COLI* SUGGESTIVE OF THE POSSIBLE ROLE OF PROTEASES IN FOLDING OF AUTOTRANSPORTER PROTEINS

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Abstract

Many of the virulence determinants secreted from pathogenic Gram-negative bacteria are autotransporter proteins, which are usually either exported to the bacterial cell surface or secreted into the external environment. There appears to be dearth of information regarding the exact mechanism of their processing into the surface-exposed passenger domains and C-terminal, β-domains. The C-terminal porin domains mediate the final step of autotransporter secretion by threading of the passenger domain through the outer membrane (OM). The native structure is formed only after this final secretion step, which does not require any energy involvement. Despite sequence divergence and functional diversity among autotransporter passenger domains almost all of them are predicted to form parallel ß-helices, indicating this structural topology may be important for secretion. The possible involvement of periplasmic environment in regulating the export of pertactin, an autotransporter passenger domain from *Bordetella pertussis* is reported.

Introduction

The autotransporters are a growing family of extracellular proteins, found in many Gram-negative bacteria that have many different functions but appear to have the same mechanism of export (Jose *et al.,* 1995; Loveless & Saier, 1997; Henderson *et al*., 1998). As the name implies, secretion through the autotransporter pathway does not require any accessory factors. The proteins containing the N-terminal signal sequence are first translocated across the cytoplasmic membrane *via* the Sec machinery. The study by Kurzchalia *et al.,* (1986) have already shown that the signal recognition particles (SRP), the soluble nucleoprotein complex, binds to hydrophobic targeting signals that directs secretion across the cytoplasmic membrane, a nonhomologous passenger domain that forms the mature virulence factor and a conserved C-terminal ~30-kDa domain that forms a ß-barrel pore in the outer membrane (OM). The diversity of functions of various autotransporter proteins in Gram-negative bacteria is associated with their passenger domains. Some of the members of this diverse family are immunoglobulin A (IgA) proteases from *Neisseria gonorrhoeae* (Klauser *et al*., 1993), and *Haemophilus influenzae* (Maurer *et al.,* 1997), the AIDA-I adhesin from *E. coli* (Suhr *et al*., 1996), IcsA from *Shigella flexneri* (Suzuki *et al*., 1995) which is involved in intracellular spread, Tsh, a temperature-sensitive hemagglutinin from an avian *E. coli* strain (Provence & Curtis 1995), Pet (Henderson & Owen, 1999) and tracheal colonization factor (Finn & Stevens, 1995), the adhesin, pertactin (Charles *et al.,* 1994) and serum resistance protein BrkA (Fernandez & Weiss, 1994) from *Bordetella pertussis*.

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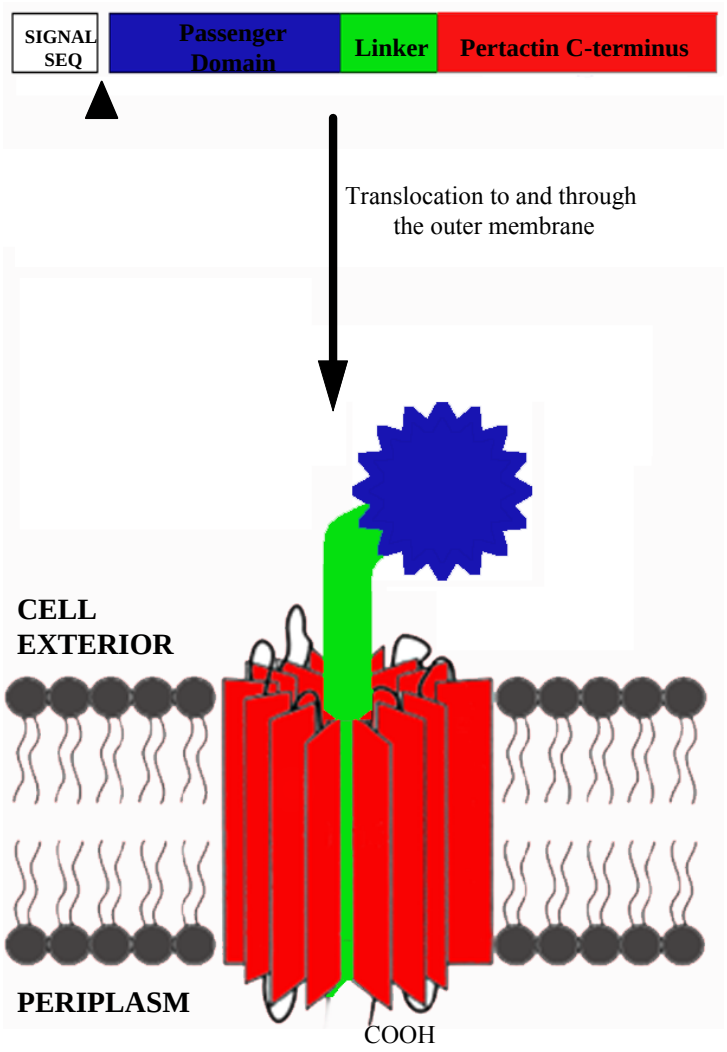

Fig. 1. Model showing the localization of mature P.69 at Gram negative Bacterial surface.

There appears to be limited information regarding the exact mechanism of their processing into the surface-exposed passenger domains and C-terminal, β-domains. Many studies in the past have indicated that the passenger domain must maintain or adopt a largely unfolded conformation to efficiently pass through the OM (Klauser *et al*., 1993; Oliver *et al.,* 2003; Henderson *et al.,* 1998). However, transport of the passenger domain in an unfolded conformation would require the passenger domain to remain in or adopt an unfolded conformation during the time required to translate, translocate and assemble the C-terminal porin in the OM (Fig. 1). In addition OM secretion is energy-independent as well as proton gradient-independent (Henderson *et al*., 2004; Thanassi *et al.,* 2005), the driving force for efficient translocation is unknown. It is possible either that they are processed by membrane-associated proteases or they may have an inherent autoproteolytic ability, which brings about their cleavage into the passenger domain and C-terminal domain.

Pertactin belongs to the family of autotransporter proteins (Henderson *et al.,* 1998; Henderson *et al.,* 2000; Henderson & Nataro, 2001) and is processed from a 93-kDa large precursor to 69- and 22-kDa proteins (Charles *et al*., 1994). The unprocessed polypeptide is directed *via* a signal peptide to the secretory machinery in the inner membrane where the signal peptide is cleaved. Subsequently, the polypeptide is directed towards the outer membrane where the 22-kDa protein is assumed to form a pore through which the 69-kDa protein is transported. After secretion *via* the autotransporter domain, proteolytic activities shape the 69-kDa protein to its final 60.37 kDa form (Gotto *et al.,* 1993). This final form (also referred to as P.69 Prn) is used in most ACVs (Miller, 1999).

The mechanism of processing of the precursor PRN (P.93) to mature PRN (P.69) and its export across the OM is not clear. The release of passenger domain from the pro-protein may occur either by auto-proteolytic cleavage as in the case of the IgA protease of *Neisseria gonorrhea*, or by the help of some of the outer membrane-localized proteases e.g., serine protease (OmpT) (Henderson *et al*., 2004). In this study we investigated the export of passenger domain (P.69) in series of *E. coli* strains lacking various envelope-associated proteases.

Materials and Methods

Bacterial strains & plasmids: The various *E. coli* protease deficient strains kindly provided by Dr. Georgiou (1991) transformed with p41869 (expressing full-length *prn*) as well as pBAD/gIIIA (expressing C-terminal *prn* + linker region) were grown routinely at 37°C on Luria Bertani (LB) broth. The pertactin gene (*prn*) (2.7 Kb) cloned into the broad host range vector pMMB66EH (7.9 Kb) was under the control of an IPTG-inducible *tac* promoter to give p41869 (kindly provided by Prof. Mark. Roberts). The pertactin C-terminal plus linker region, deduced from analysis of the *prn* sequence in GenBank (Accession No. AJ04560), (1.2 Kb) cloned into the pBAD/gIIIA, was under the control of an arabinose inducible (*araB*) promoter, and the vector also encodes a gIII signal sequence which is capable of transporting proteins *via* the Sec-dependent pathway into the periplasm promoter to give PCTlink (Fig. 2).

Expression of recombinant proteins: All the *E. coli* strains carrying pMMB66EH (control: backbone vector) or p41869 (expressing prn) as well as pBAD (control: backbone vector) or PCT1 were grown in modified LB medium supplemented with glucose 0.2% w/v and 100 µg/ml ampicillin. The expression of the recombinant PRN was induced by adding isopropyl β-D-thiogalactopyranoside (1mM IPTG; Sigma) as well as 0.2% (w/v) L-arabinose (Sigma) to the culture during the log phase (OD_{600nm} ~0.8) with incubation at 37°C continued for ~3 h. Cells were harvested at 5,000-x g for 15 min. Fractionation of bacterial cells were carried out as well as concentrated supernate samples were collected. SDS-PAGE was performed and the separated proteins (20 µg/lane) were visualized after staining with Coomassie brilliant blue. For immunoblot analysis, whole cells, the outer membrane preparations and supernate concentrates of various *E. coli* strains were separated by SDS-PAGE (12% bis-acrylamide) and transferred to nitrocellulose membrane (Hybond C, Amersham Pharmacia Biotech). The blots were processed with anti-P.69 antiserum in PBS (1:500) and horseradish peroxidase-conjugated anti-rabbit secondary antibody (Scottish Antibody Production Unit, UK) in PBS (1:1000) as well as BB05 monoclonal antisera (Linker-specific) and horseradish peroxidase-conjugated anti-mouse secondary antibody and 3', 3' diaminobenzidine (0.05% in PBS; Sigma) was used as peroxidase substrate.

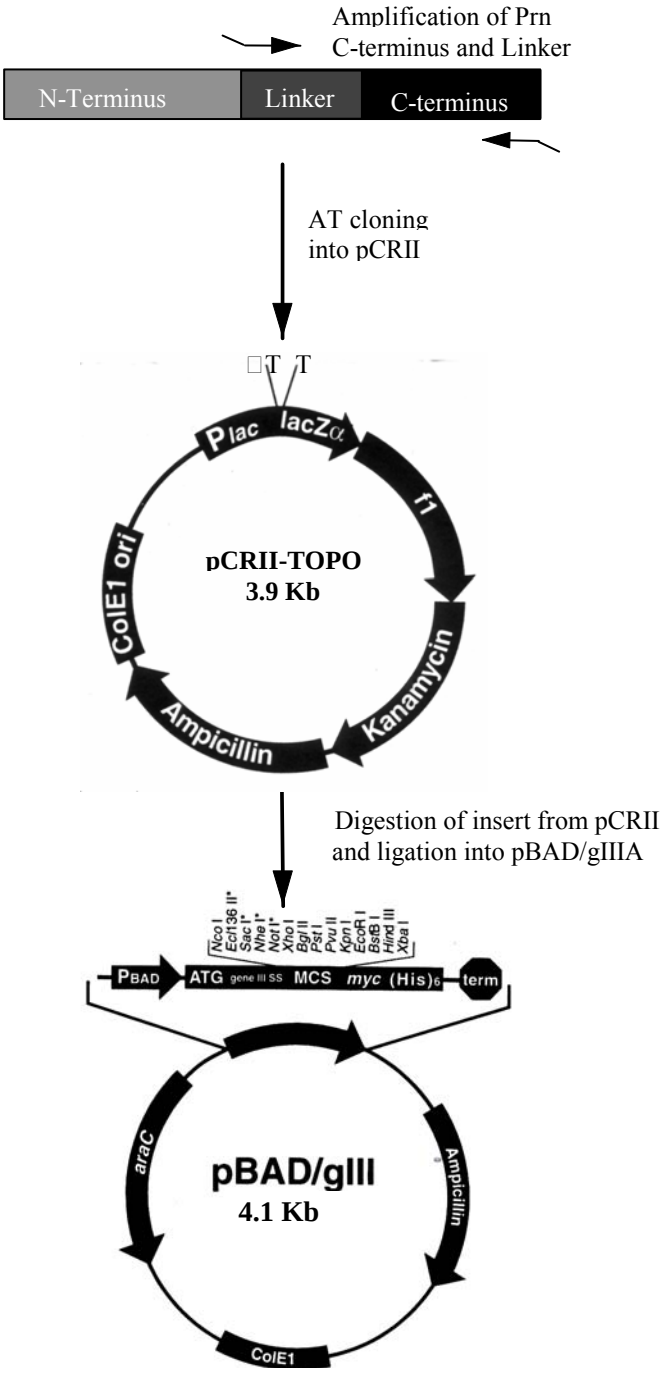

Fig. 2. Cloning o PCT and linker region in expression vector pBAD/gIIIA.

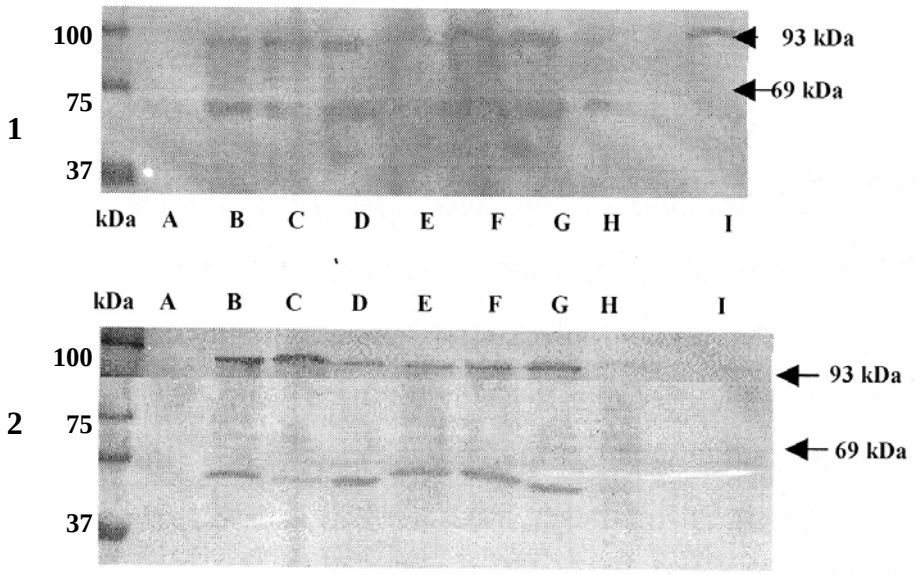

Fig. 3. Immunoblots (SDS-PAGE 12%; obtained with rabbit anti-P.69 antiserum) showing (1) whole-cell (2) outer membrane fraction and (3) concentrated supernate fraction of various protease-deficient strains expressing full length pertactin. Molecular mass standards are indicated in kDa.

Results

Autotransporter passenger domain's secretion across the outer membrane: The export of the passenger domain was investigated in *E. coli* strains lacking various combinations of envelope-associated proteases, a gift from Prof. G. Georgiou. The series of isogenic strains, deficient in number of proteases, expressing full length *prn* were analyzed for their protein profile.

The protein profiles of the whole cell of the parent strain carrying only the backbone vector pMMB66EH or expressing *prn* (p41869) as well as remaining protease-deficient strains expressing *prn* (p41869) were analyzed using anti-P.69 antiserum. P.93 specific band can be viewed in the whole cell fractions of all the proteases deficient strains expressing full-length pertactin (P.93) in the immunoblot (Fig. 3). However, AT passenger domain (P.69) also appears to be present in all strains with the exception of the whole cell fraction of most protease deficient strains of *E. coli* (HM130: Lane I). The result suggested that several protease-activities had to be absent to prevent processing, indicating that a non-specific proteolytic activity may be involved.

In order to further strengthen this observation, outer membrane profiles of various protease deficient *E. coli* strains expressing prn were run and screened with anti-P.69 antiserum. As expected the mature PRN (P.69) seems to be visible at the predicted size (69 kDa) in the outer-membrane fractions of all the strains expressing *prn*, with the exception of the most deficient strain HM130 (Fig. 3, lane 1). However, the precursor protein P.93 appears to be present in the membrane proteome of all strains expressing *prn* except that of most protease-deficient strain (Fig 3, lane 1). The other minor bands apparent in all lanes except the negative control (Fig 3; lane A) in the immunoblot are presumably the degradation products of PRN and possibly some non-specific cross-reactions.

Table 1. Summary of protein profiles in different cellular compartments of various protease-deficient strains studied.

Strains	Whole Cells kDa	OM Fractions kDa	Supernatant fractions kDa
KS272(parent) (Vector:pMMB66EH)	------	------	-----
A=KS272	$93^{++++}/69^{++++}$	$93^{+++}/69^{+++}$	69^{++}
B=SF110 *ompT*, *degp*	$93^{++++}/69^{++++}$	$93^{+++}/69^{+++}$	69^{++}
C=SF120 *ompT*, *degp*, *ptr*	$93^{++++}/69^{++++}$	$93^{+++}/69^{+++}$	69^{++}
D=HM101 *tsp*, *eda*	$93^{++++}/69^{++++}$	$93^{+++}/69^{+++}$	69^{++}
E=HM111 *ompT*, *tsp*, *eda*	$93^{++++}/69^{++++}$	$93^{+++}/69^{+++}$	69^{++}
F=HM112 *ptr*,*tsp*,*eda*	$93^{++++}/69^{++++}$	$93^{+++}/69^{+++}$	69^{++}
G=HM119 *degp*, *ompT*, *tsp*, *eda*	$93^{+++}/69^{+++}$	$93^{++}/69^{++}$	69^{+}
H=HM130 *degp*, *ompT*, *tsp*, *eda*, *ptr*	$93^{++}/69^{-}$	$93^{+}/69^{-}$	69^{-}

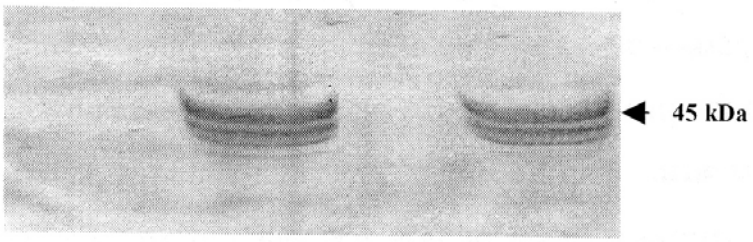

Fig. 4. Immunoblot (SDS-PAGE 12%; obtained with the monoclonal anti-PRN linker antibody BBO5) showing the whole cell fractions of *E. coli* parent and most protease deficient strains expressing the pertactin C-terminal and linker region.

The outer membrane results were further validated by looking at the concentrated (50 times) culture supernate fractions isolated from the wild type and protease-deficient strains. A summary of the results is presented in Table 1.

The results (Fig. 3) indicate that the export of the passenger domain P.69 domain appears to be affected by the periplasmic environment, perhaps requires non-specific protease or proteolytic activity, which is absent in the most protease-deficient strain HM130.

Autotransporter passenger domain is predicted to be misfolded in most protease deficient *E. coli* strain: Since a recent study indicated that despite the absence of similarity at either sequence / functional or both levels between many of the passenger domains, >97% are predicted to adopt a right-handed parallel β-helix structure, suggesting this structure may be important for AT biogenesis. *In vitro*, pertactin folds extremely slowly, perhaps because the native structure has no elements of truly local structure (i.e. α-helices). All AT OM secretion mechanisms require the passenger domain to maintain or adopt a largely unfolded conformation in the periplasm.

Periplasmic chaperones have been implicated in the proper assembly of porins in the OM (Gentle *et al*., 2004; Bulieris *et al.,* 2003), and the protease DegP has been implicated in the proper localization of the AT IcsA to the cell poles in previous study by Purdy *et al*., (2002), but no evidence exists for chaperone-mediated unfolding in the periplasm environment lacking ATP. Alternatively, a recent report suggested that the additional length possessed by a subset of ATs due to their unusually long N-terminal signal sequences may play a role in preventing misfolding of this passenger domain in the

periplasm, while it awaits secretion across the OM (Thanassi *et al.,* 2005). Since the ß-helix folds very slowly, it might not require additional cellular factors to remain unfolded in the periplasm during secretion and might explain why only a subset of ATs have unusually long signal sequences (Mirco *et al*., 2006). The extremely slow folding of the pertactin ß-helix stands in stark contrast to the microsecond-to-second folding rates reported for the majority of protein-folding studies. However, most folding studies have focused on the folding of small (often >100 aa), α-helix-rich single-domain proteins. Fast folding can play a crucial role in suppressing off-pathway aggregation by minimizing the accumulation of partially folded, aggregation-prone folding intermediates (Szabady *et al.,* 2005; Wetzel, 1996). Yet some proteins that are rich in ß-structure fold orders of magnitude more slowly than typical folding models (Engelhard & Evans 1995; Roumestand *et al*., 2001). In the case of pertactin (and by extension, other ß-helical AT proteins), slow folding may confer a functional advantage, allowing the protein to remain in a secretion-competent conformation during IM secretion and OM assembly of the C-terminal porin. Nevertheless, it remains to be determined how pertactin avoids aggregation during ß-sheet folding, particularly given the slow folding rate (Burns & Ropson, 2001).

Previous sequence comparisons and deletion studies have identified a conserved junction sequence in the extreme C terminus of some AT passenger domains that is important for efficient maturation (i.e., secretion and / or folding) of some passenger domains (Henderson *et al.,* 1998; Velarde & Nataro, 2004; Ohnishi *et al*., 1994). In pertactin, this sequence corresponds to the C-terminal two rungs of the ß-helix (Henderson *et al.,* 1998).

Results (Fig. 4) presented here further strengthen our speculation that AT passenger domain (P.69) appears to be misfolded in the periplasmic environment as the outer membrane protein profiles of the wild-type (KS272) and most deficient strains HM130 expressing only the ß-helix (30 kDa: C-terminal domain) portion and the linker region (15 kDa; involving PQP repeat region) of the PRN is exported to the OM with similar efficiency

Discussion

The AT secretion mechanism represents the most common mechanism for the secretion of virulence factors from pathogenic Gram-negative bacteria (Thanassi *et al.,* 2005). The secretion requirements, all contained within a single polypeptide chain, are astonishingly simple, in direct contrast to other mechanisms for OM secretion, which can require as many as 14 accessory proteins (Henderson *et al.,* 1998). Yet despite this simplicity, much remains unknown regarding the details of AT secretion.

The study suggested that the periplasmic environment lacking various protease activities appeared to block the secretion channels due to the accumulation of undegraded proteins in the periplasm. Therefore, P.93 which reached the outer membrane may not have been comparable to that found in the other less protease-deficient strains in terms of its quantity. Moreover, there is the possibility that P.93 would not be able to gain a correct conformation in the most protease-deficient strain which may have hindered its processing and release. This possibility was further supported by the successful insertion of the C-terminal domain with exposed linker region as seen in the outer membrane fraction of the most protease-deficient strain of *E. coli* expressing linker region and C-terminal of pertactin (PCT/linker). However, it would be more appropriate if various

domains of interest of PRN and other autotransporters could be investigated in the *B. pertussis* native system. Moreover, with the recent availability of the complete *B. pertussis* genome information, the hunt for any envelope-associated proteases of interest will become easier and simplify the investigation of their role in the processing of *B. pertussis* autotransporter proteins. In this regard, a series of protease-deficient strains of *B. pertussis* could be created (similar to those of *E. coli*) and their ability to export and release the various *B. pertussis* autotransporter proteins in the native system could be examined.

Our results do not rule out the possibility that some kind of folding is tolerated by autotransporters or even that some folding might be necessary for translocation. However, they indicate that the nature or the result of the folding of pertactin is incompatible with transport in periplasmic environment of most protease deficient strain (HM130). The simplest explanation for this interference may be that the size of the folded polypeptide blocks the translocation conduit of the autotransporter. Veiga, *et al*., (2002) hare proposed that such conduit is either a pore of 20 Å in diameter, formed upon oligomerization of multiple copies of a translocation unit (TU), or the lumen of the ß-barrel of a single TU, with a diameter around 10 Å (Muller *et al.*, 2005; Oomen *et al*., 2004; Skillman *et al*., 2005). Folded passenger AT domain due to its size appears to be incompatible with the dimensions of such channels. In that case, our experiments suggest that native passenger domains have to remain partially unfolded in order to get translocated through these narrow channels.

In conclusion, our results show, by a new approach, that periplasmic folding can interfere with translocation by autotransporters. This suggests that, in order to be translocated, native autotransporter passenger domains have to remain at least partially unfolded or that they transiently interact in a specific way with their own translocation domain or an external translocation apparatus.

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