systems. One of the systems comprises BiP and possibly PDI, and the other consists of Cnx, Crt, and ERp57. The chaperones compete for binding sites on the incoming nascent chain. Whichever binds first apparently dominates during early stages of co- and posttranslational folding. Its association delays or prevents binding of the other chaperones. Later in the folding process a transfer from one pathway to the other can, however, take place as observed for E1 here and VSV glycoprotein in a previous study (*1*).

The results indicate, moreover, that glycoproteins such as p62 and HA that possess glycans close to the NH_2-terminus enter the Cnx/Crt pathway directly without prior binding to BiP. Glycoproteins like E1 and VSV glycoprotein that have their glycans more COOH-terminally in the sequence associate first with BiP. Inhibitors of the Cnx/Crt pathway, such as castanospermine, direct proteins such as p62 and HA that normally do not bind BiP to the BiP pathway. This shows that the presence of N-linked glycans, as such, does not prevent BiP binding but rather promotes the association of the alternative chaperones. A literature survey of glycoproteins for which chaperone requirements are known is consistent with the notion that proteins with N-linked glycans within the first 50 residues do not undergo transient interaction with BiP (*14*).

Thus, in summary, our findings demonstrated that to understand the molecular basis for chaperone selection in the ER it is important to analyze growing nascent chains and to consider the precise timing of cotranslational modifications such as N-linked glycosylation and oligosaccharide trimming. Attention must be paid particularly to the location of glycosylation sites in the linear sequence.

References and Notes

1. C. Hammond and A. Helenius, *Science* **266**, 456 (1994).
2. P. Kim and P. Arvan, *J. Cell Biol.* **128**, 29 (1995).
3. I. Braakman, H. Hoover-Litty, K. R. Wagner, A. Helenius, *J. Cell Biol.* **114**, 401 (1991).
4. M. Mulvey and D. T. Brown, *J. Virol.* **69**, 1621 (1995).
5. A. Xu, A. R. Bellamy, J. A. Taylor, *J. Virol.* **72**, 9865 (1998).
6. J. Melnick, J. L. Dul, Y. Argon, *Nature* **370**, 373 (1994).
7. M. Molinari and A. Helenius, *Nature* **402**, 90 (1999).
8. W. Chen, J. Helenius, I. Braakman, A. Helenius, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 6229 (1995).
9. D. N. Hebert, B. Foellmer, A. Helenius, *Cell* **81**, 425 (1995).
10. D. N. Hebert, J.-X. Zhang, W. Chen, B. Foellmer, A. Helenius, *J. Cell Biol.* **139**, 613 (1997).
11. C. S. Copeland *et al.*, *Cell* **53**, 197 (1988).
12. S. M. Hurtley, D. G. Bole, H. Hoover-Litty, A. Helenius, C. S. Copeland, *J. Cell Biol.* **108**, 2117 (1989).
13. G. Knarr, S. Modrow, A. Todd, M. J. Gething, J. Buchner, *J. Biol. Chem.* **274**, 29850 (1999).
14. A number of cellular and viral proteins with N-linked glycans close to the NH_2-terminus have been shown to fold independently of BiP; examples follow: influenza HA [first glycan at position 8, I. Braakman *et al.*, *J. Cell Biol.* **114**, 401 (1991)], SFV p62 (13, this work), factor V [23, D. D. Pittman *et al.*, *J. Biol. Chem.* **269**, 17329 (1994)], prepro-α-factor [22, K. Su *et al.*, *J. Biol. Chem.* **268**, 14301 (1993)], Sindbis virus (SIN) PE2 [11 (*4*)], rotavirus NS28 [8, A. Xu *et al.*, *J. Virol.* **72**, 9865 (1998)], CD-mannose 6-P receptor [31, J.-X. Zhang *et al.*, *Biochem. J.* **295**, 841 (1993)]. BiP requirement for folding has been shown for proteins with glycans late in the sequence. As examples VSV glycoprotein [178 (*1*)], von Willebrandt factor [93, A. J. Dorner *et al.*, *J. Cell Biol.* **105**, 2665 (1987)], SFV E1 (141, this work), SIN E1 [139 (*4*)], HIV gp160 [88, P. L. Earl *et al.*, *J. Virol.* **65**, 2047 (1991)], amyloid precursor protein [542, Y. Yang *et al.*, *J. Biol. Chem.* **273**, 25552 (1998)], Ig heavy chain [99–160, I. G. Haas and M. Wabl, *Nature* **306**, 387 (1983)], and simian virus HA [110, D. T. Ng *et al.*, *J. Cell Biol.* **109**, 3273 (1989)].

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Convergence of the Secretory Pathways for Cholera Toxin and the Filamentous Phage, CTXϕ

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Virulence of *Vibrio cholerae* depends on secretion of cholera toxin (CT), which is encoded within the genome of a filamentous phage, CTXϕ. Release of CT is mediated by the extracellular protein secretion (eps) type II secretion system. Here, the outer membrane component of this system, EpsD, was shown to be required for secretion of the phage as well. Thus, EpsD plays a role both in pathogenicity and in horizontal transfer of a key virulence gene. Genomic analysis suggests that additional filamentous phages also exploit chromosome-encoded outer membrane channels.

Secretory systems are essential mediators of bacterial pathogenesis, for they enable release of a diverse array of bacterial virulence factors. Export of these extracellular effectors usually depends on one of four distinct multiprotein secretion systems (type I to type IV) to transport macromolecules through the inner and outer bacterial membranes (*1*). Some components of these systems probably evolved from common ancestral genes, and additional related proteins mediate pilus assembly and contribute to viral and conjugal transfer of DNA (*2, 3*). However, very few proteins are known to be integral components of more than one secretion apparatus (*4, 5*). Instead, for example, similar but distinct outer membrane pores have been shown to be essential for type II and type III secretion, type IV pilus assembly, and release of filamentous coliphages (*2*).

The genome of CTXϕ, the filamentous phage of *Vibrio cholerae* that encodes cholera toxin (CT), resembles those of the paradigmatic filamentous phages of *Escherichia coli* (e.g., f1) in many respects (*6*). However, the CTXϕ genome lacks a homolog of f1's gene IV, which encodes the outer membrane pore ("secretin"), pIV, through which f1 exits from its host (*7*). Interactions between pIV, pI (an f1-encoded inner membrane protein thought to regulate channel opening), and phage coat proteins mediate the simultaneous assembly and secretion of f1 virions (*8*). Very specific physical constraints underlie the pIV/pI interaction; thus, the secretin from the closely related coliphage Ike cannot substitute for f1 pIV (*9*). Because CTXϕ resembles f1 in that it does not lyse its host to gain release and because CTXϕ encodes proteins related to f1 phage coat proteins and assembly proteins (*6*), secretion of CTXϕ from *V. cholerae* was expected to require an outer membrane channel similar to pIV. Consequently, we investigated whether *V. cholerae* produces a pIV-like protein from a chromosomal locus that serves as a CTXϕ secretin.

A comparison of f1 pIV with the database of the *V. cholerae* genome (*10*) revealed that *V. cholerae* encodes a similar (51%) protein: EpsD, the putative outer membrane pore for the *eps* (extracellular protein secretion) type II secretion system. The *eps* apparatus is essential for secretion of CT, protease, and chitinase by *V. cholerae* (*11*). Because the CT released by infecting bacteria induces most of the symptoms of cholera, it is clear that the *eps* system plays a critical role in cholera pathogenesis (*12*). However, like other type II systems, it is only known to contribute to protein secretion.

To assess the role of *epsD* in secretion of CTXϕ, we generated Kn-insertion mutations within *epsD* in classical (O395) and El Tor (Bah-2) biotype strains of *V. cholerae* (*13*). The wild-type and mutant strains were then transformed with pCTX-Ap, the replicative form of

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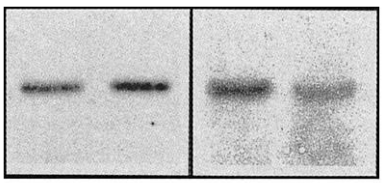

Fig. 1. Wild-type and *epsD::Kn* mutants contain comparable levels of pCTX-Ap DNA. Southern blots of Eco RI–digested plasmid DNA, which was isolated from parallel pairs of logarithmic-phase cultures, were probed with a CTXϕ-specific DNA probe to detect pCTX-Ap. Lane 1, O395/pCTX-Ap; lane 2, O395 *epsD::Kn*/pCTX-Ap; lane 3, Bah-2/pCTX-Ap; and lane 4, Bah-2 *epsD::Kn*/pCTX-Ap.

an ampicillin (Ap)–marked CTXϕ. Quantitative transduction assays revealed that the *epsD::Kn* mutant strains produced 300 times fewer CTX-Apϕ virions per cell than the corresponding wild-type strains (Table 1). Comparable levels of pCTX-Ap DNA could be detected in the wild-type and *epsD::Kn* mutant strains (Fig. 1), indicating that the reduction in virion titer was not due to impaired replication of phage DNA. Furthermore, parallel transduction experiments with O395 *epsD::Kn* and O395 as recipient strains yielded comparable numbers of Ap^R colonies (*14*), demonstrating that the *epsD* mutation did not substantially impair assembly of TCP, the type IV pilus that serves as the CTXϕ receptor. Thus, the *epsD::Kn* mutants did not suffer from a global secretory defect. Instead, EpsD appears to be specifically required for CTXϕ secretion.

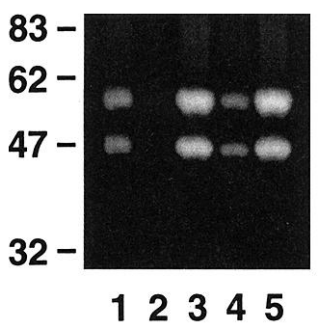

Fig. 2. Bah-2 *epsD::Kn* secretes a reduced amount of hemagglutinin/protease. Supernatants from early stationary phase cultures were subjected to SDS–polyacrylamide gel electrophoresis on a 10% polyacrylamide gel containing 0.1% gelatin. The gel was washed two times for 45 min in 2.5% Triton X-100 and for 30 min (room temperature) and 2 hours (37°) in tris-buffered saline + 5 mM $CaCl_2$ and then stained with Coomassie Blue. Lane 1, strain 3083; lane 2, HAP-1, a 3083 derivative containing an inactivated form of the gene encoding hemagglutinin/protease (*hap::Kn*) (*16*); lane 3, Bah-2/pCTX-Ap; lane 4, Bah-2 *epsD::Kn*/pCTX-Ap; and lane 5, Bah-2 *epsD::Kn*/pCTX-Ap/pGZ119:EpsD. Numbers at left indicate molecular weight markers.

We also characterized protein secretion by the *epsD::Kn* mutants. As expected, GM-1 enzyme-linked immunosorbent assays (ELISAs) revealed that O395 *epsD::Kn* secreted less than 10% of the toxin it produced (Table 1), substantially less than the 95% secreted by wild-type O395, but similar to the amount secreted by other *eps* mutant strains (*11*). CT secretion by derivatives of the nontoxigenic strain Bah-2 (*15*) could not be assessed; however, a zymogram of culture supernatants revealed that Bah-2 *epsD::Kn*/pCTX-Ap secreted less hemagglutinin/protease, the major *V. cholerae* secreted protease (*16*), than did its wild-type counterpart (Fig. 2). Thus, EpsD plays its predicted role in protein secretion as well as mediating export of CTXϕ.

To confirm that the secretory defects of the *epsD::Kn* strains were linked to the *epsD* mutations, we assessed the impact of plasmid-encoded EpsD on secretion by the mutant strains (*17*). The *epsD* plasmid complemented the *epsD* mutation: It enabled the El Tor and the classical mutant strains to secrete wild-type levels of virions and partially restored secretion of proteins (Table 1 and Fig. 2). In contrast, introduction of the control vector lacking *epsD* into the *epsD::Kn* strains did not influence CTX-Apϕ or protein secretion.

Although secretion of CTXϕ through alternate membrane channels may account for the residual CTXϕ produced by our *epsD::Kn* mutants, it is more likely that these rare transducing particles were detected because the *epsD::Kn* mutation does not completely inactivate *epsD*. The Kn^R cassette was inserted only 30 amino acids from the COOH-terminus of EpsD (*13*); thus, a mutant protein might still form minimally functional outer membrane channels. Several earlier attempts to generate *epsD* alleles with different disruptions were unsuccessful (*14*), and recent work indicates that EpsD deficiency greatly impairs growth of *V. cholerae* (*18*). Consequently, we believe that our *epsD::Kn* mutant strains are not entirely devoid of functional EpsD and that their residual EpsD activity enables bacterial survival, some secretion of *eps* protein substrates (Fig. 2), and a very low level of CTXϕ secretion.

The failure of plasmid-encoded EpsD to fully restore secretion of CT by O395 *epsD::Kn* may reflect an altered level of EpsD relative to the 11 other proteins produced from the *eps* gene cluster. In previous studies of the additional *eps* genes, comparable incomplete complementation has been observed (*11*). However, plasmid-encoded EpsD restored CTXϕ titers to wild-type levels, suggesting that *eps* genes other than *epsD* are not required for phage secretion. To test this hypothesis, we assayed phage production by a previously described *epsE* mutant, O395 *epsE::Kn* (*11*). O395 *epsE::Kn*/pCTX-Ap yielded virion titers exceeding those produced by wild-type O395/pCTX-Ap (6.8×10^5 $A^{600^{-1}}$ ml^{-1} versus 1.2×10^5 $A_{600}{}^{-1}$ ml^{-1}, where A_{600} is absorbance at 600 nm), despite secreting only 9% of synthesized toxin. Similarly, a Bah-2 derivative that produced a dominant negative EpsE (*19*), which blocks protein secretion, still efficiently secreted virions. This strain, Bah-2/pCTX-Kn/pMS43, yielded slightly more virions (4.6×10^7 $A^{600^{-1}}$ ml^{-1}) than did Bah-2/pCTX-Kn/pMS42 (4.2×10^7 $A_{600}{}^{-1}$ ml^{-1}), which overexpressed wild-type EpsE (*20*). Thus, EpsE, and by inference a functional *eps* secretory structure, is not necessary for efficient CTXϕ production. Furthermore, the failure of *epsD::Kn* strains to secrete CTXϕ virions is not an indirect result of the secretory and membrane changes observed in *eps* mutant strains.

Table 1. CTX-Apϕ and CT secretion by wild-type and *epsD::Kn* strains of *V. cholerae*. Data are representative of assays performed at least three times. NA, not applicable.

Strain*	Phage titer (CTX-Apϕ ml^{-1} $A_{600}{}^{-1}$†)	CT secreted‡ (%)
O395	NA	>95%
O395 *epsD::Kn*	NA	<10%
O395/pCTX-Ap	1.2×10^5	>95%
O395 *epsD::Kn*/pCTX-Ap	4.1×10^2	<10%
O395 *epsD::Kn*/pCTX-Ap/pGZ119:EpsD	5.4×10^5	40%
O395 *epsD::Kn*/pCTX-Ap/pGZ119HE	3.3×10^2	<10%
Bah-2/pCTX-Ap	3.2×10^5	NA
Bah-2 *epsD::Kn*/pCTX-Ap	9.4×10^2	NA
Bah-2 *epsD::Kn*/pCTX-Ap/pGZ119:EpsD	1.5×10^5	NA
Bah-2 *epsD::Kn*/pCTX-Ap/pGZ119HE	1.2×10^2	NA

*O395 contains two chromosomal copies of *ctxAB*; CT measured in the ELISAs is produced from these genes, not pCTX-Ap. pGZ119:EpsD contains an IPTG-inducible *epsD*. †Transduction assays were performed as previously described (*6*); logarithmic-phase donor cultures were grown at 37°C in LB broth with antibiotics and 1 mM IPTG. Phage titer per ml of supernatant was normalized to the A_{600} of the donor culture. Titers are average results based on at least three experiments. ‡Toxin secretion was measured with GM1 ELISAs as previously described (*26*), except that cultures were grown overnight at 30°C in LB broth with antibiotics and 1 mM IPTG. Cell lysis or leakage (assessed with β-lactamase assays) was comparable for all strains and did not contribute substantially to release of toxin (*14*).

We propose the following model for CTX phage secretion. CTXϕ's homolog of f1 pI, Zot, a presumed inner membrane protein (*21*), interacts with a multimer of the outer membrane protein EpsD and thereby induces opening of this outer membrane channel, through which the phage is released. Additional interactions between Zot, EpsD, and phage coat proteins are also likely; interactions between CTX phage-encoded proteins and proteins of the *eps* apparatus other than EpsD are probably not required. It is not known whether a single EpsD multimer can interact simultaneously with components of both secretory pathways or whether the phage and protein secretory processes compete for access to the outer membrane channel.

Phage exploitation of a host secretin has not been demonstrated previously; however, analyses of the genomes of additional filamentous phages suggest that reliance upon a chromosome-encoded secretin may be a common strategy for phage secretion. Within the GenBank database, there are at least five filamentous phages other than CTXϕ—fs1, Vf12, Vf33, Cf1c, and Pf1—that do not appear to encode a pIV homolog and thus may rely upon a chromosomal protein instead. All these phages infect bacterial species that contain type II secretion systems. In contrast, the filamentous coliphages that encode a phage-specific secretin infect a host that generally does not produce a secretory apparatus (*22*). Thus, coliphages may have been constrained during their evolution to rely upon a phage-encoded secretin. Alternatively, phage-encoded secretins may grant access to a broader range of host species or confer some other evolutionary advantage.

It is somewhat surprising that EpsD can mediate both CTXϕ and CT secretion. Most secretins are unable to function within heterologous systems, even systems composed of very similar proteins with highly related substrates (*9, 23*). Furthermore, the two secretory processes to which EpsD contributes are markedly different. Phage export releases a cytoplasmic DNA molecule coated with inner membrane–derived coat proteins, whereas type II secretion systems export only free, periplasmic proteins. Nonetheless, in *V. cholerae*, these two disparate classes of secretion substrates both appear to pass through an outer membrane pore composed of EpsD. The convergence of phage and protein secretion pathways may be a clue that structurally similar periplasmic complexes are assembled during each process. Indirect evidence in support of this hypothesis has already been provided by the findings that both pathways bear similarities, at either the sequence or structural level, to type IV pilus assembly (*2*). Our finding that a filamentous phage and a type II secretion apparatus use the same secretin provides additional evidence that the two export systems have a common evolutionary origin and suggests that they may still maintain mechanistic similarities.

References and Notes

1. B. B. Finlay and S. Falkow, *Microbiol. Mol. Biol. Rev.* **61**, 136 (1997).
2. M. Russel, *J. Mol. Biol.* **279**, 485 (1998).
3. P. J. Christie, *J. Bacteriol.* **179**, 3085 (1997).
4. R. Binet, S. Letoffe, J. M. Ghigo, P. Delepelaire, C. Wandersman, *Gene* **192**, 7 (1997).
5. K. J. Fullner and J. J. Mekalanos, *Infect. Immun.* **67**, 1393 (1999).
6. M. K. Waldor and J. J. Mekalanos, *Science* **272**, 1910 (1996).
7. D. K. Marciano, M. Russel, S. M. Simon, *Science* **284**, 1516 (1999).
8. M. Russel, *Trends Microbiol.* **3**, 223 (1995).
9. ———, *J. Mol. Biol.* **231**, 689 (1993).
10. We searched the *V. cholerae* database with the Institute for Genome Research's BLAST Search Engine for Unfinished Microbial Genomes (www.tigr.org/cgi-bin/BlastSearch/blast.cgi?).
11. M. Sandkvist *et al.*, *J. Bacteriol.* **179**, 6994 (1997).
12. J. B. Kaper, J. G. Morris Jr., M. M. Levine, *Clin. Microbiol. Rev.* **8**, 48 (1995).
13. The targeting vector is described by A. Ali *et al.*, *Infect. Immun.* **68**, 1967 (2000).
14. B. M. Davis, E. H. Lawson, M. Sandkvist, M. K. Waldor, H. H. Kimsey, unpublished data.
15. G. D. Pearson, A. Woods, S. L. Chiang, J. J. Mekalanos, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 3750 (1993).
16. C. C. Hase and R. A. Finkelstein, *J. Bacteriol.* **173**, 3311 (1991).
17. *EpsD* (base pairs 1159 to 3245 of the *eps* gene cluster: GenBank accession number L33796) was cloned into the Cm^R, isopropyl-β-D-thiogalactopyranoside (IPTG)–inducible plasmid pGZ119HE (*24*).
18. M. Judson and J. J. Mekalanos, unpublished data.
19. pMS43 and pMS42 are equivalent to pMS27 and pMS12 (*25*), except that they are Cm^R.
20. pCTX-Kn is a more stable replicon than pCTX-Ap; this probably accounts for the consistently higher phage titers from strains containing pCTX-Kn. Transduction assays were performed as described in Table 1.
21. E. V. Koonin, *FEBS Lett.* **312**, 3 (1992).
22. A. P. Pugsley and O. Francetic, *Cell Mol. Life Sci.* **54**, 347 (1998).
23. M. Lindeberg, G. P. Salmond, A. Collmer, *Mol. Microbiol.* **20**, 175 (1996).
24. M. Lessl *et al.*, *J. Bacteriol.* **174**, 2493 (1992).
25. M. Sandkvist, M. Bagdasarian, S. P. Howard, V. J. DiRita, *EMBO J.* **14**, 1664 (1995).
26. M. Sandkvist, L. P. Hough, M. M. Bagdasarian, M. Bagdasarian, *J. Bacteriol.* **181**, 3129 (1999).

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Functional Role of Caspase-1 and Caspase-3 in an ALS Transgenic Mouse Model

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Mutations in the copper/zinc superoxide dismutase (SOD1) gene produce an animal model of familial amyotrophic lateral sclerosis (ALS), a fatal neurodegenerative disorder. To test a new therapeutic strategy for ALS, we examined the effect of caspase inhibition in transgenic mice expressing mutant human SOD1 with a substitution of glycine to alanine in position 93 ($mSOD1^{G93A}$). Intracerebroventricular administration of zVAD-fmk, a broad caspase inhibitor, delays disease onset and mortality. Moreover, zVAD-fmk inhibits caspase-1 activity as well as caspase-1 and caspase-3 mRNA up-regulation, providing evidence for a non–cell-autonomous pathway regulating caspase expression. Caspases play an instrumental role in neurodegeneration in transgenic $mSOD1^{G93A}$ mice, which suggests that caspase inhibition may have a protective role in ALS.

ALS is a neurodegenerative disorder involving motor neuron loss in the brain, brainstem, and spinal cord and resulting in progressive paralysis. ALS is universally fatal, with an average mortality of 5 years after onset (*1*). Familial ALS accounts for 10 to 20% of all cases; the remaining cases are sporadic. Both forms of the disease have indistinguishable clinical and histopathological features (*2*). Mutations of the SOD1 (mSOD1) gene have been identified in some cases of familial ALS (*3, 4*). Transgenic mice have been generated expressing different mSOD1 genes identified in ALS patients (*5, 6*). Like humans with ALS, these mice develop an adult-onset progressive motor deterioration universally leading to early death and have been used as models for the disease (*5, 7*). Although the mechanisms leading to motor neuron degen-

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