The chaperone-assisted membrane release and folding pathway is sensed by two signal transduction systems

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The assembly of interactive protein subunits into extra-cellular structures, such as pilus fibers in the Enterobacteriaceae, is dependent on the activity of PapD-like periplasmic chaperones. The ability of PapD to undergo a β zipper interaction with the hydrophobic C-terminus of pilus subunits facilitates their folding and release from the cytoplasmic membrane into the periplasm. In the absence of the chaperone, subunits remained tethered to the membrane and were driven off-pathway via non-productive interactions. These off-pathway reactions were detrimental to cell growth; wild-type growth was restored by co-expression of PapD. Subunit misfolding in the absence of PapD was sensed by two parallel pathways: the Cpx two-component signaling system and the σ E modulatory pathway.

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Introduction

Periplasmic chaperones are an essential component of the machinery required for biogenesis of surface-localized structures such as bacterial pili (Hultgren *et al.*, 1991, 1993, 1996, and references therein). These structures are typically associated with virulence, since they contain adhesins which mediate binding to complementary surfaces of host receptors (Hultgren *et al.*, 1993, 1996; Roberts *et al.*, 1994). P pili are produced specifically by uropathogenic strains of *Escherichia coli* and have been studied extensively as a model system to understand macromolecular assembly (Hultgren *et al.*, 1991, 1993, 1996). Subunits destined for assembly into pili pass through a periplasmic intermediate state (Hultgren *et al.*, 1991, 1996). Stability of pilus subunits in the periplasm is dependent on the activity of the PapD chaperone, which binds to each of the P pilus subunits to form bimolecular complexes (Hultgren *et al.*, 1991, 1996). Periplasmic complexes between PapD and PapA, PapE, PapF, PapG and PapK have been identified and characterized (Hultgren *et al.*, 1991, 1993, 1996; unpublished data). Once formed, chaperone-subunit complexes are targeted to outer membrane assembly sites, composed of proteins known as ushers, where pilus biogenesis takes place (Dodson *et al.*,

1993). In the absence of the chaperone the subunits collapse into off-pathway aggregates that are proteolytically degraded (Bullitt *et al.*, 1996; Hultgren *et al.*, 1996). The studies described in this report provide new insights into the mechanism of how the chaperone guides translocation of subunits across membranes and facilitates their folding and assembly into pili.

PapD is the prototype member of a family of 26 periplasmic chaperones present in most members of the Enterobacteriaceae as well as in other bacteria (Hultgren *et al.*, 1996; Hung *et al.*, 1996). The 3-dimensional structure of PapD revealed that it possesses two globular immunoglobulin-like domains oriented such that a deep cleft is formed between the domains (Kuehn *et al.*, 1993). The structural basis for part of the chaperone-subunit interaction was solved by co-crystallizing PapD with a peptide corresponding to the C-terminus of PapG (Kuehn *et al.*, 1993).

Utilizing an *in vitro* assay we demonstrate that PapD facilitates the targeting of pilus subunits into the periplasm and drives the subunits down a productive pathway such that the subunits are kinetically partitioned away from an aggregative fate. We present data suggesting that PapD facilitates the final tertiary fold of the PapG adhesin. We also discovered that misfolded subunits activate one or both signal transduction pathways (Mecbas *et al.*, 1993; Danese *et al.*, 1995) that induce synthesis of multiple factors, such as the DegP protease, for recruitment to the periplasm. One pathway operates via a classic two component regulatory system, Cpx (Danese *et al.*, 1995; Danese and Silhavy, 1997; Pogliano *et al.*, 1997), while the second pathway results in direct modulation of a σ factor, σ^E (Mecbas *et al.*, 1993; Raina *et al.*, 1995; Rouviere *et al.*, 1995; De Las Penas *et al.*, 1997; Missiakis and Raina, 1997; Missiakis *et al.*, 1997).

Results

An assay to study the role of PapD in pilus biogenesis

Synthesis of P pilin subunits in the absence of PapD results in their proteolytic degradation (Hultgren *et al.*, 1989; Slonim *et al.*, 1992). We employed an isogenic pair of strains KS272 (MC1000) and KS474 (KS272 *degP::kan*) to test the role of the periplasmic protease DegP (Strauch *et al.*, 1989; Lipinska *et al.*, 1990) in degradation of subunits. It was anticipated that in this genetic background new insights into the mechanism of action of PapD could be gained, since subunits would accumulate in the absence of the chaperone and be more amenable to study.

In KS272 high level synthesis of PapG and PapE in the absence of PapD caused a profound growth defect, whereas PapA and PapK were tolerated when produced

Fig. 1. Subunit synthesis is toxic, resulting in a severe growth defect. (A) PapA synthesis is toxic in KS474 (pHJ2, filled squares) but tolerated in the parent strain KS272 (pHJ2, filled circles). (B) PapG synthesis is toxic in both KS272 and KS474 (pHJ8, filled symbols). (C) PapK synthesis is not toxic in either KS272 or KS474. (D) PapE synthesis is toxic (pHJ13, open circles) and toxicity is suppressed by co-synthesis of PapD (pHJ13+pHJ203, open squares). (E) Non-aggregative PapA derivatives PapA-G150T (pHJ33, open circles), PapA-Y162L (pHJ34, filled triangles) and PapA-C22S (pHJ35, dashed line) are non-toxic in KS474. (F) Removal of the signal sequence (pFS4A, filled squares) renders PapA non-toxic in KS474.

at similar or higher levels than PapE or PapG (Figure 1A–C and unpublished data). In KS474 PapA synthesis was found to be highly toxic, while PapK was tolerated

when synthesized at similar or higher levels (Figure 1A and C). As expected from the results in KS272, PapG and PapE were highly toxic in KS474 (Figure 1B and D).

Subunit synthesis (PapG, PapE and PapA) resulted in growth arrest; affected cultures showed no change in viable counts ($\sim 1 \times 10^7$ c.f.u./ml throughout the 6 h assay) when plated on LB agar lacking IPTG (unpublished data). We hypothesize that interactive surfaces on subunits promote non-productive interactions in the absence of the chaperone, leading to growth arrest. The inability of PapK synthesis to inhibit growth may be related to its inability to self-associate (Bullitt *et al.*, 1996), which makes it less aggregative. The reason for PapG toxicity is not readily apparent. However, this protein is larger than the other pilins and previous studies have revealed a potential for it to aggregate *in vitro* (Kuehn *et al.*, 1991; see also below).

Toxicity is a result of exposure of the subunit β zipper motif and other interactive surfaces

The conserved C-terminus of subunits has been implicated in forming an interactive surface that drives subunit-subunit interactions (Bullitt *et al.*, 1996). A Leu substitution at Tyr162 (Y162L) and a Thr substitution at Gly150 (G150T) in the C-terminal β zipper motif of PapA have been shown to abolish the subunit-subunit interactions necessary for formation of pilus rods (Bullitt *et al.*, 1996). However, both the mutant and wild-type proteins accumulated in the periplasmic space of both strains, KS272 and KS474, to the same extent when co-synthesized with the PapD chaperone (Bullitt *et al.*, 1996). A Ser substitution for Cys22 (C22S) in PapA, which forms a conserved disulfide with Cys61, also abolished the subunit-subunit interactions necessary for pilus formation (R.Striker and S.J.Hultgren, personal communication), but was stable when expressed in strain KS474. Since these mutations disrupt interactive surfaces on the subunit, we tested whether these mutations would also abolish the toxic effect associated with expression of the subunit in the absence of the chaperone. Figure 1E shows that the non-aggregative PapA mutants (Y162L, G150T and C22S) are non-toxic when expressed in KS474, whereas a conserved substitution, Y162F, which assembles into pili, although to a lesser degree, remains toxic. These data suggest that exposure of the C-terminal β zipper in the periplasm causes the observed toxic phenotype in strain KS474.

Toxicity arises from the periplasm

The growth defect resulting from subunit production in the absence of PapD suggests that the toxic effect originates from the periplasm. Further support for this hypothesis was garnered by demonstrating that signal sequence processing is required for the observed toxicity. A PapA mutant lacking a signal sequence is not toxic when expressed in KS474 (Figure 1F). These results suggest that engagement of the Sec machinery and transport across the inner membrane is required for toxicity.

Toxicity suppression and correlation with complex formation

If the role of the periplasmic chaperone is to protect interactive subunit surfaces and block their aggregation, then production of the chaperone along with the toxic subunit should be sufficient to suppress toxicity. In both strains, KS272 and KS474, co-expression of the periplasmic chaperone PapD suppressed the toxicity of

Fig. 2. Subunit toxicity is relieved by co-synthesis of the PapD periplasmic chaperone. (A) PapG toxicity (pHJ31, open circles) is suppressed by co-synthesis of PapD (pL5101, filled circles). (B) PapA toxicity (pHJ2, open circles) is suppressed by co-synthesis of PapD (pHJ9203, filled circles) but not by co-synthesis of R8G PapD mutant protein (pHJ9204, open squares).

pilin subunit synthesis (Figures 1D, E, 2A and B and unpublished data).

The invariant residues Arg8 and Lys112 in the crevice of the PapD cleft form a critical part of the subunit binding site (Slonim *et al.*, 1992; Kuehn *et al.*, 1993). Substitutions at Arg8 and Lys112 had no effect on PapD accumulation in the periplasm (Slonim *et al.*, 1992; Kuehn *et al.*, 1993). We tested the effect of mutating the invariant Arg8 residue to Gly (R8G) on the ability of PapD to suppress the toxic effects of PapA. The R8G mutation in PapD blocked its ability to suppress PapA toxicity (Figure 2B). These data confirm that the interactions, via the cleft residues, known to be essential for chaperone-subunit complex formation are also required for suppression of toxicity.

Periplasmic localization is dependent on PapD

In the light of precedents established with other systems, several explanations can be offered for the toxicity caused by overproduction of the Pap subunits. For example, it is possible that high level production of these subunits causes a deleterious 'jamming' of the general secretion machinery. This would inhibit envelope biogenesis and cause accumulation of the precursor forms of secreted proteins in the cytoplasm (Snyder and Silhavy, 1992). We ruled out the 'jamming' hypothesis by showing that the kinetics of signal sequence processing for periplasmic maltose binding protein (MBP) were unaffected by induction of either PapA or PapG (unpublished data). We also ruled out the related hypothesis that overproduced Pap subunits might interfere with discharge of proteins from the secretion machinery by showing that MBP release into the periplasm was also unaffected (unpublished data). These results demonstrate that overproduction of Pap subunits does not interfere with the function of the general protein secretion machinery.

The toxicity of overproduced Pap subunits could also result from improper targeting of these proteins to the periplasm (Matsuyama *et al.*, 1995). Accordingly, we tested the periplasmic localization of pilin subunits when expressed in KS474 in the absence or presence of PapD (Figure 3A). Production of PapA, PapE, PapG and PapK in the absence of PapD resulted in poor accumulation of the subunits in the periplasm. These results suggest that efficient release of pilin subunits into the periplasm is dependent on PapD-subunit interactions.

The above results suggest that subunits synthesized alone are either not produced efficiently or are associated with another compartment of the cell, such as the inner membrane. The fate of subunits expressed in the absence of the chaperone was investigated by fractionating KS474/pHJ8 (*papG*)/pHJ9203 (*papD*) into periplasmic and inner membrane fractions (Figure 3B). The fractions were immunoblotted with polyclonal antisera directed against the PapD-PapG complex. As shown in Figure 3B, PapG is only detectable in the periplasm following co-induction of PapD synthesis; PapG is associated with the inner membrane fraction when produced in the absence of PapD. Similar results were obtained with PapA and PapE (unpublished data). We conclude that the chaperone facilitates release of the subunit from an inner membrane location to the soluble periplasmic compartment.

Spheroplast assay for testing the targeting function of PapD

To further define the role of the chaperone in mediating subunit compartmentalization we established a semi-*in vitro* assay utilizing spheroplasts. A related spheroplast system was employed by Matsuyama *et al.* (1995) to define the activity of the periplasmic lipoprotein carrier protein P20. In our system spheroplasts were prepared from KS474/pHJ8; the spheroplasts were induced and pulse labeled in the presence or absence of purified PapD added to the supernatant. As shown in Figure 4A, addition of purified PapD to the spheroplast suspension increased the amount of immunoprecipitable PapG in the supernatant by >30-fold (Figure 4A, compare lanes 1 and 2).

In a separate experiment PapD was added 10 or 30 min following the chase to test if targeting required that PapD

Fig. 3. Targeting of pilin subunits to the periplasmic space is dependent on PapD. (A) Periplasmic fractions prepared from KS474 strains producing pilin subunits alone (odd numbered lanes) or producing subunits and PapD (even numbered lanes). Coomassie blue staining of SDS-PAGE clearly demonstrates that accumulation of stable pilin subunits PapA, PapE, PapG and PapK is dependent on the chaperone. (B) Western blot with anti-PapD-PapG antisera demonstrating PapD-mediated targeting of PapG to the periplasmic space. Fractionation of KS474/pHJ8/pHJ9203 reveals that PapG resides in the inner membrane fraction when synthesized in the absence (lane 4) of PapD, but is efficiently targeted to the periplasmic space by PapD (lane 2). In lanes 3 and 5 the samples were not exposed to β -mercaptoethanol prior to SDS-PAGE, demonstrating that cysteine disulfides are formed in PapG prior to interaction with PapD.

interact with PapG during or shortly after translocation. In this experiment PapD-facilitated targeting would require that PapD act on post-translocated PapG that had accumulated in the inner membrane. PapD was fully capable of releasing PapG into the soluble fraction 10 and 30 min post-chase (Figure 4A, lanes 4 and 5). Furthermore, trypsin treatment of spheroplasts producing PapG released an ~25 kDa fragment of PapG; demonstrating that a portion of PapG is exposed on the surface of the spheroplast (unpublished data). These findings suggest that membrane-associated PapG is stable and a substrate for PapD and we conclude that membrane-associated PapG is an intermediate in the biogenesis pathway.

FimC, HifB and SfaE, the periplasmic chaperones required for assembly of type 1, *Haemophilus* and S pili respectively, and the newly discovered EcpD (*E. coli* PapD)

Fig. 4. Spheroplast release assay. PapG synthesis was induced in spheroplasts produced from KS474/pHJ8 by addition of 1 mM IPTG. (A) Prior to pulse labeling with ^{35}S Trans label (cysteine + methionine), 7 μg purified PapD (lane 2), the R8A PapD derivative (lane 3) or purified histidine-tagged PapD (lane 6) were added and allowed to incubate for 10 min. Following a 10 min chase with unlabeled cysteine + methionine the spheroplast suspension was subjected to centrifugation to remove the spheroplasts and the supernatant immunoprecipitated with antibody containing reactivity to PapG. In lanes 4 and 5 purified PapD was added 10 (lane 4) or 30 (lane 5) min following the cold chase. (B) The F314S substitution in PapG reduces the efficiency of targeting relative to wild-type PapG (compare lanes 3 and 5). Both the double mutant F314S+G302V and deletion of the C-terminal 100 amino acids relieve PapG dependence on PapD for efficient partitioning to the supernatant fraction.

(Raina *et al.*, 1993) were purified and tested in the spheroplast assay. None of these chaperones were able to stimulate release of PapG into the supernatant fraction (unpublished data).

The structural basis of the targeting reaction was investigated by testing the effect of site-directed mutations in both PapD and PapG of residues that are known to be critical for PapD–PapG complex formation based on the co-crystal structure of PapD with the PapG C-terminal peptide (Kuehn *et al.*, 1993). Arg8 forms a critical part of the invariant subunit binding site in the chaperone cleft. The targeting activity of PapD was severely compromised

by an R8A substitution, arguing that the invariant cleft is critical for this function (Figure 4A, lane 3). Phe314 and Gly302 are two conserved C-terminal PapG residues that are part of the conserved β zipper motif that is recognized by PapD. The Phe was changed to Ser (F314S PapG) as a single mutation and it was also combined with a Gly mutation to Val at position 302 (F314S+G302V PapG) as part of a double mutation. The C-terminal 100 residues of PapG were also deleted, yielding the PapG Δ 100 truncate. The F314S mutation in PapG reduced the efficiency with which PapD released this derivative into the supernatant (Figure 4B, lane 5). F314S+G302V PapG was

efficiently released to the supernatant in the absence of PapD, in contrast to wild-type PapG (Figure 4B, lanes 6 and 7). Moreover, the presence of PapD had no effect on release of this double mutant protein. Similarly, deletion of the C-terminal 100 amino acids resulted in a stable 24 kDa PapG derivative that was released into the supernatant independent of PapD (Figure 4B, lanes 8 and 9). These results argue that the β zipper region of a subunit facilitates its retention in the inner membrane in the absence of PapD and that binding of PapD to this surface is required for release from the membrane.

Overproduction of PapG activates a two component signal transduction system

Since the Cpx and σ^E modulatory systems both respond to overproduction of extracytoplasmic proteins (Mecbas *et al.*, 1993; Danese *et al.*, 1995; Danese and Silhavy, 1997; De Las Penas *et al.*, 1997; Missiakos and Raina, 1997; Missiakos *et al.*, 1997), we wished to determine if these systems were also affected by overproduction of the PapE and PapG subunits. Accordingly, we transformed strains PND2000 (MC4100, *degP-lacZ*), SP558 (PND2000, *cpxA::cam*) and SP559 (PND2000, *cpxR:: Ω*) with pHJ8 (overproducing PapG) and pMMB66 (vector control for pHJ8) and then determined the amount of *degP-lacZ* transcription generated from these transformants. Lanes 1 and 2 of Figure 5A show that overproduction of PapG stimulates *degP-lacZ* transcription 6.7-fold. Interestingly, overproduction of PapG only stimulates *degP-lacZ* transcription 2.4-fold in the absence of CpxA (compare lanes 3 and 4 of Figure 5A). Finally, in a *cpxR*⁻, *cpxA*⁻ background (SP559) the stimulatory effect of PapG overproduction on *degP-lacZ* transcription is only 2.2-fold (compare lanes 5 and 6 of Figure 5A). Thus overproduction of PapG stimulates *degP* transcription, in part, by activation of the Cpx signal transduction system.

The results presented in Figures 1 and 2 indicated that co-synthesis of the PapD chaperone blocks the growth defect associated with overproduction of P pilin subunits. Thus we hypothesized that co-synthesis of PapG and PapD would suppress PapG-mediated induction of *degP* transcription. As shown in Figure 5B, production of PapG with simultaneous synthesis of PapD significantly reduced the amount of *degP* transcription generated from PND2000. This result implies that *E. coli* monitors the assembly status of PapG and uses this information to modulate *degP* transcription.

PapG synthesis stimulates σ^E activity

Figure 5A shows that synthesis of PapG causes a residual induction of *degP* transcription even in the absence of the Cpx pathway (compare lanes 1 and 2 with lanes 5 and 6). Therefore, a second signal transduction system must also sense the unfolded state of PapG and stimulate *degP* transcription. The σ^E modulatory system has been shown to activate both the *degP* and the *rpoHP3* promoters (Mecbas *et al.*, 1993). Because the *rpoHP3* promoter is solely controlled by σ^E and not by the Cpx system (Danese *et al.*, 1995), we used an *rpoHP3-lacZ* operon fusion to determine if PapG synthesis also stimulated σ^E activity. Figure 5C shows that PapG synthesis (from pHJ8) stimulates *rpoHP3-lacZ* [SP616 (MC4100, *rpoHP3-lacZ*)] transcription 2.4-fold, which closely agrees with the ~2.2-fold

stimulation of *degP* transcription in the absence of the Cpx proteins (Figure 5A, lanes 5 and 6).

Production of PapG stimulates transcription from a second Cpx-regulated locus

In an effort to corroborate the conclusion that synthesis of unchaperoned PapG stimulates the Cpx pathway, we assayed a second Cpx-regulated locus, *cpxP*, whose transcription is wholly dependent on CpxR and is independent of σ^E (P.N. Danese, doctoral thesis, Princeton University). To this end, we determined the β -galactosidase activities of strains SP594 (MC4100, *cpxP-lacZ*) and SP620 (SP594, *cpxR:: Ω*) following induction of PapG synthesis. As shown in Figure 5D, PapG synthesis in the parental strain (SP594) stimulates *cpxP* transcription 10.3-fold. In a *cpxR*⁻, *cpxA*⁻ background (SP620) *cpxP* transcription is not induced following PapG synthesis.

PapE synthesis stimulates Cpx but not σ^E activity

We also performed the assays described above with the P pilus fibrillar component PapE (pHJ13). Surprisingly, unlike PapG, PapE production stimulates only the Cpx signal transduction pathway. As shown in Figure 5E, induction of PapE synthesis in PND2000 caused a 4-fold stimulation of *degP* transcription (compare lanes 1 and 2), whereas in SP558 and SP559 the amount of *degP-lacZ* transcription is not elevated above the background level (compare lanes 3 and 4 and lanes 5 and 6). High level synthesis of PapE also stimulated *cpxP-lacZ* transcription in a Cpx-dependent fashion (Figure 5F) and failed to stimulate transcription from the *rpoHP3-lacZ* fusion (unpublished data). Thus while the Cpx pathway can monitor the level of both PapE and PapG, the σ^E modulatory system can only monitor PapG levels.

The Cpx pathway as a monitor of pilus assembly

We also observed a significant activation of *degP-lacZ* transcription when the entire P pilus operon was expressed in the absence of the PapD chaperone (Figure 5G, compare lanes 1, 2 and 3). This P pilus-mediated induction of *degP* transcription is significantly reduced in the absence of the Cpx pathway (compare lanes 2 and 3 with lanes 8 and 9); however, a residual induction in *degP* transcription was still detected (compare lanes 7–9). This residual induction (~2.1-fold) appears to be due to an increase in σ^E activity because high level expression of the P pilus operon also stimulated *rpoHP3-lacZ* transcription ~2.1-fold (Figure 5H). The implication from these results is that the Cpx and σ^E modulatory systems monitor the assembly status of the P pilus subunits and use this information to modulate synthesis of the periplasmic protease DegP in response to this input.

Chaperone folding assay

The presence of misfolded or partially denatured proteins in the periplasm is thought to be a signal that leads to activation of *degP* transcription. PapD is known to be required to stabilize pilus subunits, so we decided to investigate further the folded state of pilin subunits synthesized in the absence of PapD. PapG activity can be monitored *in vitro* by binding to the Gal α (1-4)Gal digalactoside (galabiose) receptor (Hultgren *et al.*, 1991, 1996). Lectin binding activity has been shown to be a

Fig. 5. Pilus subunit synthesis in the absence of PapD results in promoter activation via the Cpx and σ^E signal transduction pathways. (A) *degP-lacZ* promoter activation as a result of PapG production utilizes both the Cpx and σ^E modulation pathways. *degP-lacZ* activity was monitored in strains PND2000, SP558 and SP559 following induction of synthesis of PapG from pHJ8. (B) Co-synthesis of PapD abrogates *degP-lacZ* promoter activation. *degP-lacZ* activity was monitored in PND2000 following induction of PapG synthesis (pHJ9208) or PapG and PapD synthesis (pHJ9208 + pHJ6). (C) Synthesis of PapG activates the *rpoHP3-lacZ* promoter, indicating that PapG misfolding is sensed by the σ^E modulatory system. *rpoHP3-lacZ* activity was monitored in SP616 following induction of PapG synthesis (pHJ8). (D) PapG activation of the *cpxP-lacZ* promoter is Cpx dependent. *cpxP-lacZ* activity was monitored in SP594 and SP620 following induction of PapG synthesis (pHJ8). (E) Synthesis of PapE activates the *degP-lacZ* promoter in a Cpx-dependent manner. *degP-lacZ* activity was monitored in PND2000, SP558 and SP559 following induction of PapE synthesis (pHJ13). (F) The *cpxP-lacZ* promoter is activated by PapE synthesis and is Cpx dependent. *cpxP-lacZ* activity was monitored in SP594, SP619 and SP620 following induction of PapE synthesis (pHJ13). (G) Pilus biogenesis is monitored by the Cpx and σ^E signal transduction pathways. *degP-lacZ* activity was monitored in PND2000, SP558 and SP559 following induction of pilus synthesis from pFJ29 (*pap*) and pFJ29-71 (*papD*⁻). *degP-lacZ* promoter activation is seen, especially following induction of the pilus operon lacking a functional *papD* gene (pFJ29-71, *papD*⁻). (H) *degP-lacZ* promoter activation resulting from initiation of pilus biogenesis is not entirely Cpx dependent, as seen in triggering of the σ^E modulatory pathway. *rpoHP3-lacZ* activity was monitored following induction of the pilus operon from pFJ29 and pFJ29-71 in SP616.

function of tertiary structure (Weis *et al.*, 1988). We tested the ability of periplasmically localized full-length PapG to bind to galabiose when produced alone or together with wild-type PapD. Nearly 100% of the immunoprecipitable PapG in the periplasm bound to digalactoside when PapD

was present. However, when PapD was absent <10% of the immunoprecipitable periplasmic PapG bound to receptor (Figure 6). These results indicate that in the absence of PapD, PapG is unable to achieve or be maintained in a native-like receptor binding conformation.

Fig. 6. PapD-mediated folding of PapG as assayed by galactose chromatography. (A) Labeled periplasms containing PapG only (lanes 1 and 7) or PapG along with PapD (lanes 2 and 8), PapD R8A (lanes 3 and 9), PapD K112A (lanes 4 and 10), FimC (lanes 5 and 11) or the PapD-FimC chimera (lanes 6 and 12) were subjected to immunoprecipitation (lanes 1–6) or galactose–Sepharose chromatography (lanes 7–12).

(B) Phosphorimager quantification of lanes 1–6 of the gel in (A). (C) Quantification of binding efficiency of PapG when expressed alone or in the context of chaperones and chaperone mutants, expressed as a percentage of immunoprecipitable material (A, lanes 1–6) that bound to galactose–Sepharose (A, lanes 7–12).

The ability of PapD to maintain PapG in a receptor binding conformation depends on the invariant chaperone anchor

The R8A and K112A mutations in PapD, which reduced the ability of the chaperone to facilitate PapG targeting, also failed to assist PapG to fold or be maintained in a native-like receptor binding conformation, since periplasmic PapG was unable to bind receptor (Figure 6A, lanes 3 and 4, and C). Similarly, FimC was unable to stimulate PapG targeting to the periplasm and apparently unable to guide the protein down a productive folding pathway (Figure 6A, lane 5, and C). A PapD–FimC chimeric chaperone was constructed by genetically fusing domain 1 (encoding residues 1–112) of *papD* to domain 2 (encoding residues 112–205) of *fimC*; this protein is stable in the periplasm (unpublished data). The chimera was unable to mediate the assembly of either type 1 or P

pili (unpublished data), however, in contrast to FimC, it was able to suppress the toxicity associated with PapA expression (unpublished data). The chimera also partially stimulated PapG targeting to the periplasm; however, the resulting periplasmic PapG was unable to bind receptor (Figure 6).

Discussion

The assembly of a family of extracellular structures, including pili, afimbrial adhesins and proteinaceous capsules, in bacteria is a conserved process that utilizes a family of periplasmic chaperones (Hultgren *et al.*, 1991, 1993, 1996; Hung *et al.*, 1996). Bacteria face a multitude of problems when assembling extracellular structures, including: (i) translocation of monomeric proteins across two membranes and their transport through the highly

concentrated periplasm; (ii) prevention of spontaneous assembly in membranes and/or the periplasm; (iii) blocking of off-pathway processes that lead to misfolding, aggregation and proteolysis. Periplasmic chaperones, along with cognate outer membrane ushers, constitute a novel molecular machinery (Hultgren *et al.*, 1991, 1993, 1996) necessary for guiding biogenesis of adhesive organelles in Gram-negative bacteria. Pilus subunit proteins cross the inner membrane via the Sec pathway (Dodd *et al.*, 1984; Pugsley and Possot, 1994); as all subunits have a classic signal sequence that is processed to form the mature pilin in the periplasm (Dodd *et al.*, 1984; Hultgren *et al.*, 1991, 1993, 1996; unpublished data). However, we discovered that the Sec machinery does not efficiently release pilus subunits into the periplasm. The appearance in the periplasm of soluble pilin subunits PapA, PapE, PapG and PapK is greatly facilitated by interaction with the periplasmic PapD chaperone. In the absence of PapD, PapG was found to be associated with the inner membrane. Mutagenesis studies combined with *in vitro* and *in vivo* studies demonstrated that targeting of subunits into the periplasm was dependent on an anchoring interaction of the terminal carboxylate group of the subunits to the invariant Arg8 and Lys112 residues in the crevice of the chaperone cleft. In addition to this molecular anchoring interaction, β zippering of the conserved C-terminus of the subunits along the exposed edge of the G1 β -strand of PapD also facilitated release of the subunits from the cytoplasmic membrane into the periplasm. Interestingly, it is the presence of the hydrophobic C-terminal motif that makes subunit targeting dependent on PapD: this motif tethers the subunits to the cytoplasmic membrane. Deletion of the C-terminal 100 residues of PapG renders periplasmic localization independent of PapD. Mutations in many of the conserved alternating hydrophobic residues in the C-terminal β zipper motif of PapG were found to behave similarly to the PapG Δ 100 protein, suggesting that altering a feature of the β zipper (hydrophobicity) or the conformation of the C-terminal domain results in a protein that behaves as if the entire C-terminal domain is missing (unpublished data). This same C-terminal motif has been shown, in the case of PapA, to be part of an interactive surface that participates in subunit-subunit interactions required for assembly of pilus rods (Bullitt *et al.*, 1996). Premature subunit-subunit interactions are prevented in the periplasm by PapD, which binds to and caps this surface (Kuehn *et al.*, 1993; Bullitt *et al.*, 1996). Our current studies have shown that the ability of PapD to release subunits from the membrane also depends upon this interaction.

It remains to be determined whether PapG is associated with Sec components or a novel site in the inner membrane prior to its interaction with PapD. However, since the function of the general secretion machinery is not impaired in any way in the absence of PapD, we favor membrane association. The C-terminal β zipper motif is extremely hydrophobic (Kuehn *et al.*, 1993; Hung *et al.*, 1996) and may insert into the membrane in a manner similar to a classic stop transfer sequence (Kuroiwa *et al.*, 1991). Alternatively, the C-terminus of the subunits may be part of an edge strand of a β -sheet that 'lies' on top of the membrane, inserting its alternating hydrophobic sidechains into the membrane. In either case, we suggest that the

membrane-associated state may represent an intermediate in pilus biogenesis, functioning to protect the C-terminal interactive surface and prevent it from participating in non-productive interactions. A spheroplast assay showed directly that purified PapD added to whole spheroplasts facilitated release of PapG from the surface into the supernatant in a process that can occur long after synthesis is complete. The ability of PapD to partition fully translocated PapG supports the hypothesis that the membrane-associated form of PapG represents a stable intermediate in biogenesis.

Our studies are reminiscent of those recently reported by Matsuyama *et al.* (1995) describing the function of the P20 protein, which acts as a carrier protein for assembly of LPP (major outer membrane lipoprotein) into the outer membrane. Using a spheroplast system these investigators demonstrated that P20 facilitated release of LPP from the inner membrane into the soluble fraction. LPP is thought to be tethered to the cytoplasmic membrane by the lipid portion of the lipoprotein. The interaction of P20 with LPP results in its release from the membrane and formation of a specific complex between LPP and P20.

A pilus subunit in a membrane-associated state most likely does not exist in a native conformation. *In vivo* in the absence of PapD, subunits are susceptible to proteolytic degradation by the DegP protease, which is thought to act on denatured or partially unfolded substrates (Kolmar *et al.*, 1996). In a *degP*⁻ strain and in the absence of PapD the majority of PapG protein fractionates with the cytoplasmic membrane, although some can be recovered in the periplasm. Less than 10% of the PapG protein recovered from periplasmic extracts in these cells is capable of binding to the Gal α (1-4)Gal receptor. In contrast, when co-expressed with the PapD chaperone nearly 100% of the recoverable PapG protein binds to receptor. This result argues that the chaperone-assisted release reaction may drive folding of a subunit to completion and/or be necessary to maintain the subunit in a native conformation. The membrane-bound state may represent a molten globule or a highly folded intermediate with partially denatured local structures. Based on circular dichroism and Chou-Fasman predictions, there is evidence that pilin monomers are predominantly β -sheet proteins (unpublished results). Thus the chaperone could provide a predefined context in the form of a template for initial β -strand formation and thereby predispose subsequent formation of β -sheet structure.

PapD may facilitate membrane release and folding of subunits via a two-step mechanism. Previous studies have demonstrated that PapD recognizes a 'second site' on PapG separate from the C-terminal β zipper (Xu *et al.*, 1995). Our current hypothesis is that the second site is exposed in the membrane-associated state and is recognized by PapD, possibly via domain 2 (Figure 7). Presumably, residues in domain 2 of PapD also participate in driving the folding process, since a PapD-FimC chimera containing domain 1 of PapD and domain 2 of FimC was unable to guide PapG into a receptor binding conformation. This binding event may trigger a conformational change in the subunit that results in exposure of its C-terminus and allows its partitioning from a membrane-associated state to an extended strand along the G1 β -strand of PapD.

Fig. 7. A model of signal transduction pathways that monitor pilus biogenesis. The chaperone-mediated protein translocation, folding and targeting pathway is illustrated (see text for details). Off-pathway products of this pathway result in activation of one or both of the Cpx and σ^E modulatory signal transduction pathways. Triggering the Cpx pathway results in activation of the σ^E -dependent promoter *degP* and four σ^E -independent promoters (Danese *et al.*, 1995; Danese and Silhavy, 1997; Pogliano *et al.*, 1997). Triggering the σ^E modulatory pathway via RseAB results in up-regulation of the *degP* promoter as well as three others (Mecbas *et al.*, 1993; Raina *et al.*, 1995; Rouviere *et al.*, 1995; De Las Penas *et al.*, 1997; Missiakis and Raina, 1997; Missiakis *et al.*, 1997). Activation of the Cpx pathway by NlpE (Snyder *et al.*, 1995) and the σ^E modulatory pathway by OMP expression (Mecbas *et al.*, 1993) are also depicted.

This two-step pathway explains how membrane release is coupled to template-assisted folding (Figure 7).

Interestingly, the PapK subunit is not toxic in KS474; however, this subunit is still dependent on the chaperone for periplasmic targeting. Previous studies (Hultgren *et al.*, 1993, 1996) have shown that PapK is present in the pilus in single or low copy and that it does not aggregate into oligomeric sub-assemblies *in vitro* (Bullitt *et al.*, 1996; unpublished data). This finding suggests that the aggregation potential of subunits is, in part, a cause of toxicity. The properties of the β zipper C-terminal motif that increase the aggregation potential of a subunit may be the same properties that contribute to inner membrane retention, since inner membrane-localized PapK has not been demonstrated (unpublished data). This proposal is supported by the finding that PapA mutants that fail to multimerize and/or are not assembly competent (Bullitt *et al.*, 1996) are also non-toxic. Although PapG is believed to exist in single or very low copy in the pilus (Kuehn *et al.*, 1992) it remains a highly toxic protein. Earlier work showed that PapG diluted out of urea formed an aggregate that failed to enter an isoelectric focusing gel, suggesting that PapG aggregates can form under certain conditions (Kuehn *et al.*, 1991). PapG is also larger (35 kDa) than the other pilins (13-17 kDa) due to the

presence of the N-terminal Gal α (1-4)Gal binding domain. We suggest that toxicity is related to specific properties in the C-terminal β zipper that influence inner membrane retention, as well as other hydrophobic surfaces that may be exposed during folding or in the membrane-retained state, but the precise cause of toxicity is not clearly understood.

Our results show that off-pathway products of pilus biogenesis require new gene products to be synthesized and recruited to the periplasm to prevent toxicity. There are at least two pathways that monitor the assembly state of extracytoplasmic proteins (Mecbas *et al.*, 1993; Cosma *et al.*, 1995; Danese *et al.*, 1995; Raina *et al.*, 1995; Danese and Silhavy, 1997; De Las Penas *et al.*, 1997; Missiakis and Raina, 1997; Missiakis *et al.*, 1997; Figure 7). The Cpx two-component and the σ^E modulatory pathways respond to different extracytoplasmic signals and each pathway uses these external stimuli to activate *degP* transcription in addition to several other genes (Danese and Silhavy, 1997; De Las Penas *et al.*, 1997; Missiakis and Raina, 1997; Missiakis *et al.*, 1997). In this study we have shown that PapG synthesis stimulates *degP* transcription by activating both the Cpx and σ^E modulatory pathways. PapE synthesis only results in activation of *degP* via the Cpx pathway. Co-production of PapD suppresses

transcriptional activation, suggesting that the signal sensed in the periplasm is misfolding or aggregation of the subunits. However, the signal could also result from membrane perturbation due to PapG association with the inner membrane. In either case, these non-productive associations appear to be triggered by the C-terminal zipper. If the subunit β zipper is not correctly positioned along the exposed edge of the G1 β -strand of PapD it apparently engages in the non-productive associations that stimulate *degP* transcription.

The precise mechanism of activation of the Cpx and σ^E modulatory pathways is unclear (Danese and Silhavy, 1997; De Las Penas *et al.*, 1997; Missiakis and Raina, 1997; Missiakis *et al.*, 1997). It appears that these systems evolved to ameliorate perturbations in certain protein assembly processes by up-regulating the synthesis of protein folding agents, such as DsbA (Danese and Silhavy, 1997; Pogliano *et al.*, 1997) and peptidyl-prolyl isomerases (Missiakis *et al.*, 1996; Danese and Silhavy, 1997; Pogliano *et al.*, 1997), and proteases, like DegP, that can destroy misfolded proteins. We suggest that, in terms of pilus biogenesis, the Cpx and σ^E modulatory pathways may function to monitor pilus assembly and use this information to control synthesis of extracytoplasmic proteases and protein folding agents that can ultimately regulate the assembly process. Furthermore, these two signal transduction pathways could also conceivably be used to transmit signals relevant to pathogenesis, such as signaling attachment to the host epithelium.

Materials and methods

Strains and genetic constructs

KS272 {MC1000 [F⁻ Δ (*ara-leu*)7697 *galE galK* Δ *lacX74 rpsL* (*str^r*)]} and the isogenic protease-deficient strain KS474 (KS272 *degP::kan*) were kindly provided by J.Beckwith and have been previously described (Strauch and Beckwith, 1988; Strauch *et al.*, 1989). DH5 α [*supE44* Δ *lacU169* (Φ 80 *lacZ*AM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] (Hanahan, 1983) was used in all cloning steps and in expression studies with *papG*. Other constructs used were PND2000 (MC4100, λ RS88[*degP-lacZ*] (Danese *et al.*, 1995), SP558 (PND2000, *cpxA::cam*), SP559 (PND2000, *cpxR:: Ω*), SP616 (MC4100, λ RS45 [*rpoHP3-lacZ*] (Mecenas *et al.*, 1993), SP594 (MC4100, λ RS88 [*cpxP-lacZ*] (P.N.Danese, doctoral thesis, Princeton University), SP619 (SP594, *cpxA::cam*) and SP620 (SP594, *cpxR:: Ω*). pMMB66 was used as an expression plasmid to place subunits under control of the P_{tac} promoter. pHJ2 was constructed by moving the *papA*-containing *Bam*HI–*Eco*RI fragment from pPAPAY162 (a gift of M.-J.Wick, Washington University) into pMMB66 (Furste *et al.*, 1986). pHJ8 was constructed by moving the *Bam*HI–*Eco*RI fragment, containing *papG*, from pPAP55 (Hultgren *et al.*, 1989) into pMMB66. pHJ13 was constructed by moving the *Bam*HI–*Eco*RI fragment, containing *papE*, from pPAP63 (Lindberg *et al.*, 1989) into pMMB66. pTK5 was constructed by cloning the *Bam*HI–*Sal*I fragment, containing *papK*, from pFJ11 (Jacob-Dubuisson *et al.*, 1993) into pMMB66. The arabinose promoter plasmid pMON6235 was kindly provided by M.Obukowicz (Monsanto Corporate Research, St Louis, MO). pMON6235 Δ cat was created by digesting pMON6235 with *Nco*I and *Hind*III (removing the *cat* gene) and religating the large fragment. pHJ9 was constructed by cloning a spectinomycin cassette (kindly provided by M.Caparon, Washington University) into the *Eco*RI and *Pvu*II sites of pMON6235 Δ cat, truncating the resident *amp* gene. *papD* was cloned under the control of the P_{ara} promoter in pHJ9 in two steps. First, *papD* was moved as a *Bam*HI–*Eco*RI fragment from pLS101 (Slonim *et al.*, 1992) into pIC20H, creating pHJ3. *papD* was then moved from pHJ3 as a *Bam*HI–*Bgl*II fragment into pHJ9, creating pHJ9203. Point mutant derivatives in PapD (pHJ9204 carries the R8A derivative of PapD) were moved into pHJ9 following a similar protocol. pHJ23, containing a C-terminal truncated version of *papG*, was previously described (Xu *et al.*, 1995). pHJ7 (F314S) and pHJ30 (G302V+F314S)

were constructed using the *duf⁻ ung⁻* protocol essentially as described previously (Slonim *et al.*, 1992). pLSR8A and pK112A have been previously described (Slonim *et al.*, 1992; Kuehn *et al.*, 1993). Mutants were constructed using the *duf⁻ ung⁻* protocol essentially as described previously (Zoller and Smith, 1983). pHJ31 was constructed by restricting pHJ21 with *Bam*HI and *Eco*RI and filling in the ends with Klenow fragment. The blunt fragment was then cloned into the *Sma*I site of pTRC99A, placing the *papG* gene under control of the *trc* promoter. pHJ9208 was constructed in two steps. First, the *papG*-containing *Bam*HI–*Eco*RI fragment was moved from pHJ8 into pUC19, creating pHJ21. The *papG*-containing *Bam*HI–*Bgl*II fragment was then moved from pHJ21 into pHJ9, creating pHJ9208. The PapD–FimC chimera was constructed by cloning a PCR product containing domain 2 of FimC into *Hind*III- and *Bam*HI-restricted pLS101, replacing domain 2 of PapD. pRS3A and pRS4A carry the wild-type and leaderless versions of *papA* respectively in pTRC99A (R.T.Striker, unpublished data). pHJ33, pHJ34 and pHJ35, carrying the G150T, Y162L and C22S mutations in *papA* respectively, have been previously described (Bullitt *et al.*, 1996). pFJ29 and pFJ29-71, containing the whole *pap* operon and the *papD* null allele respectively, have been previously described (Jacob-Dubuisson *et al.*, 1994).

Toxicity assay

Overnight cultures, grown at 30°C in the presence of appropriate antibiotics, were diluted 100-fold into Luria broth containing appropriate antibiotics and inducers. Toxicity was achieved with three of the subunits studied using different levels of isopropyl- β -D-thiogalactoside (IPTG). PapG toxicity required 80 μ M IPTG, while toxicity of PapA required 0.3 mM IPTG. PapE was toxic when induced at 35 μ M IPTG, while PapK was non-toxic at 0.5 mM IPTG. Arabinose was added to the culture at 4% at the onset of growth to induce PapD and PapD mutants in the pHJ9 vector for suppression of PapA toxicity. pHJ31 (*papG*) and pHJ31/pLS10 (*papG*, *papD*) were used to demonstrate PapG toxicity and suppression by PapD, due to an apparent suppressant effect of arabinose precluding the use of pHJ9203 (*papD*) for induction of PapD by arabinose addition. This suppressant effect of arabinose was observed to a lesser degree with PapE toxicity, but was not observed with PapA toxicity and suppression with PapD induced from pHJ9203. Following a 2 h lag, the OD₆₀₀ of the cultures was monitored at hourly intervals. Growth was followed for at least 6 h. Viable counts (c.f.u.) were plated at each time point, revealing that 'toxicity' was a manifestation of a failure of the culture to grow following subunit induction, i.e. 1×10^7 c.f.u. at time 0 and 6 h later, whereas in the presence of the chaperone the c.f.u. reached $>1 \times 10^9$ over the growth period.

Chaperone and subunit induction and bacterial fractionation

Bacterial cultures were grown in Luria broth in the presence of appropriate antibiotics at 30°C to an OD₆₀₀ of 0.6, at which time IPTG was added to 0.5 mM, α -D-arabinose to 0.2% and growth allowed to proceed for 60 min. Fractionation of bacteria was performed as previously described (Dodson *et al.*, 1993). Briefly, periplasm was isolated from equivalent gram quantities of bacteria by the sucrose/lysozyme method (Jones *et al.*, 1993). Following periplasm isolation, cytoplasm and membrane fractions were obtained by sonication followed by centrifugation. The membrane pellet was fractionated into inner and outer membrane fractions by differential solubility in sarkosyl (Dodson *et al.*, 1993).

Spheroplast release assay

Spheroplasts were produced following the procedure of Witholt *et al.* (1976) as follows. KS474/pHJ8 was grown in minimal medium A with 0.2% glycerol overnight and diluted 1/10 into fresh minimal medium A. At an A₆₀₀ of 0.8 the bacteria were harvested by centrifugation and resuspended in 1 ml 0.2 M Tris–HCl, pH 8. The cell suspension was then diluted by 1/2 into 0.2 M Tris–HCl, pH 8, 1 M sucrose, 0.5 mM EDTA. Lysozyme was added to 60 μ g/ml final concentration. The suspension was again diluted 2-fold with dH₂O and incubated at 25°C for 20 min. Spheroplast formation was monitored by light microscopy, with $>95\%$ spheroplast formation occurring within 15 min. Spheroplasts were stabilized by addition of 0.02 M MgCl₂. The spheroplasts were collected by centrifugation and resuspended in minimal medium A plus 0.02 M MgCl₂. To assay the partitioning of PapG from the spheroplast suspension, 1 mM IPTG was added and incubation allowed to proceed for 5 min. The suspension was then pulsed with ³⁵S Trans label (cysteine + methionine) (ICN, Costa Mesa, CA) for 1 min, followed by a 10 min unlabeled chase with 0.1 M cysteine + methionine. Purified PapD (2–10 μ g) was added at either the IPTG induction point or following a 10–30 min cold chase. The spheroplast pellet was removed by centrifugation

and the supernatant subjected to immunoprecipitation with anti-Pap tip antisera (Medimune Inc., Gaithersburg, MD), which has strong reactivity against PapG. The immunoprecipitations were quantitated with a Phosphorimager (BioRad, Hercules, CA) and Molecular Analyst software (BioRad).

β -galactosidase assays

Bacteria were grown overnight in Luria broth, subcultured (1:50) and grown for 60 min, at which point IPTG was added to 0.5 mM to induce pilus subunit proteins. The induced strains were allowed to grow for 90 min and then harvested for β -galactosidase assay. β -galactosidase activities were determined utilizing a microtiter plate assay (Slauch and Silhavy, 1991) and expressed as $(U/A_{600}) \times 10^3$, where $U = \mu\text{mol product formed/min}$. A minimum of four independent assays were performed on each strain and averaged. pHJ8 (*papG*) and pHJ13 (*papE*) were used to demonstrate promoter activation following induction of synthesis by IPTG addition. pHJ208 (*papG*) and pHJ6 (*papD*) were used to demonstrate suppression of promoter activation by PapG synthesis.

Gal α (1-4)Gal chromatography

The PapD–PapG complex was purified from periplasmic fractions on Gal α (1-4)Gal–Sephacrose beads as previously described (Hultgren *et al.*, 1989). For the folding studies ^{35}S -labeled periplasm (see below) was applied to Gal α (1-4)Gal–Sephacrose beads in 1 M NaCl, 1 $\times$ PBS (0.12 M NaCl, 2.7 mM KCl, 0.067 M Na_2HPO_4 , 0.014 M KH_2PO_4 , pH 7.4) for 30 min at 25°C. Following extensive washing with 1 M NaCl, 1 $\times$ PBS the bound material was eluted with soluble Gal α (1-4)Gal (17 mg/ml) in 1 M NaCl, 1 $\times$ PBS by gentle rocking for 15 min at 25°C.

Other methods

SDS-PAGE, isoelectric focusing and Western blot analysis were performed as previously described (Slonim *et al.*, 1992; Dodson *et al.*, 1993; Jones *et al.*, 1993; Xu *et al.*, 1995). Pulse/chase of periplasmic contents was performed essentially as described previously (Stader *et al.*, 1986; Snyder and Silhavy, 1992). Bacteria were diluted, from an overnight culture in minimal medium A, 1/10 into fresh minimal medium A and allowed to grow to an A_{600} of 0.8, at which time the culture was induced with 1 mM IPTG for 15 min. An aliquot of 1 ml of the induced culture was then pulsed with ^{35}S Trans label (cysteine + methionine) for 1 min and chased for 10 min with 0.1 M unlabeled cysteine + methionine. Labeling was stopped by dilution of the label mixture into ice-cold 50 mM Tris–HCl, pH 8, 100 $\mu\text{g/ml}$ chloramphenicol. Following periplasm preparation, immunoprecipitation was carried out with anti-Pap tip antisera (Medimmune Inc., Gaithersburg, MD), which contains strong reactivity against PapG.

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