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Outer-membrane PapC molecular usher discriminately recognizes periplasmic chaperone–pilus subunit complexes

(pathogenesis/bacterial adhesin/postsecretional assembly/P pili)

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ABSTRACT **P pili are highly ordered composite structures consisting of thin fibrillar tips joined end-to-end to rigid helical rods. The production of these virulence-associated structures requires a periplasmic chaperone (PapD) and an outer membrane protein (PapC) that is the prototype member of a newly recognized class of proteins that we have named "molecular ushers." Two *in vitro* assays showed that the preassembly complexes that PapD forms with the three most distal tip fibrillar proteins (PapG, PapF, and PapE) bound to PapC. The relative affinity of each complex for PapC was found to correlate with the final position of the subunit type in the tip fibrillum. In contrast, the complexes PapD forms with the major component of the pilus rod, PapA, or the pilus rod initiating protein, PapK, did not recognize PapC. The *in vitro* data argue that differential targeting of chaperone–subunit complexes to PapC may be part of a mechanism to ensure the correctly ordered assembly of adhesive composite pili.**

Microbial attachment frequently mediated by a stereochemical fit between adhesin molecules and complementary host-cell surface receptors is an important early event in colonization and infection of a host by pathogenic microorganisms (1, 2). Uropathogenic *Escherichia coli* present a Galα(1–4)Gal binding adhesin, PapG, at the distal tip of P pili. P pili are highly ordered composite fibers (3) consisting of a thin fibrillar structure that is joined end-to-end to a helical rod-like structure composed mainly of PapA (4), the major pilin (5, 6). The tip fibrillum is composed mostly of repeating PapE subunits (3) but also contains the initiator/adaptor proteins PapF and PapK (6, 7). PapF is required to correctly join PapG to PapE and is involved in initiating the formation of the tip fibrillum. The incorporation of PapK terminates the growth of the tip fibrillum and is involved in nucleating the formation of the pilus rod (7). PapH appears to be located at the base of the pilus rod and plays a role in the termination of pilus growth and in anchoring the pilus to the cell wall (8). The deduced order of the structural proteins from the distal end of the composite pilus is thus PapG, PapF, PapE, PapK, PapA, and finally PapH.

The production of P pili requires two assembly genes encoding PapD and PapC. PapD is a 28.5-kDa periplasmic chaperone that binds to nascently translocated pilus subunits, importing them into the periplasmic space in assembly-competent conformations. This binding seems to drive the correct folding and assembly of the subunits by preventing incorrect interactions that cause aggregation and proteolytic degradation (1, 9–11). PapC is an 86-kDa outer membrane protein (12). In the absence of PapC, the PapD chaperone remains bound to subunits in chaperone–subunit complexes, thus preventing pilus assembly. We hypothesized that a mechanism must exist whereby chaperone–subunit complexes are targeted to outer membrane assembly sites containing PapC, where pilus proteins are dissociated from the chaperone and incorporated into the growing pilus. In this study, using two different *in vitro* systems, we show that PapC differentially recognizes the various chaperone–subunit complexes, and we suggest a strategy to ensure that every pilus rod is joined end-to-end to an adhesive tip fibrillum.

MATERIALS AND METHODS

Plasmid Construction. Standard molecular techniques (13) were used to subclone *papC* into the polylinker of pMMB66 (16). A kanamycin-resistance gene bracketed by inverted polylinkers (14) was inserted into an *Ssp* I-linearized pPAP5 (5, 15) plasmid at base pair position 2821 (8), near the end of the *papH* gene. This plasmid was called pKD14. The *papC* gene was then subcloned from pKD14 as a *Xho* I–*Hin*dIII fragment into pMMB66, creating pKD100. pKD101 was created by an insertion of a kanamycin-resistance gene into pKD100.

Induction and Partial Purification of PapC. The *papC* gene was induced in cultures of either HB101 or ORN103 carrying either pKD100 or pKD101 with 0.1–10.0 mM isopropyl thiogalactoside (IPTG). PapC was extracted from the bulk of the outer membrane prepared as described (17) by three successive freeze (−20°C)/thaw treatments in water followed by heating of the membrane preparation to 37°C for 30 min. After centrifugation at 27,000 × *g* for 30 min, >50% of the PapC protein was found in the membrane supernatant, while the bulk of the membrane lipid and other proteins were in the pellet (see Fig. 1*B*).

Preparation of Periplasmic Extracts. Periplasmic extracts were prepared as described (18). The concentrations of Pap proteins in the periplasmic extracts were determined by SDS/PAGE followed by Coomassie blue staining and densitometry with an LKB Ultroscan densitometer (the known concentration of the added lysozyme was an internal standard). When indicated, periplasmic extracts were diluted with periplasm extraction buffer (20% sucrose/20 mM Tris/5 mM EDTA/20 mM $MgCl_2$).

Purification of PapD, PapD–PapG, PapD–PapK, PapD–PapA, and Tip Fibrillae. PapD was purified by ion-exchange chromatography (10). PapD–PapG complex and tip fibrillae were purified by Galα(1–4)Gal affinity chromatography as described (7, 9). PapD–PapA and PapD–PapK complexes were purified by ion-exchange and hydrophobic interaction chromatography (R.T.S., F.J.-D., and S.J.H., unpublished data).

Poly(vinylidene difluoride) (PVDF)-Immobilized PapC Recognition Assay. Membrane extracts with PapC were subjected to SDS/PAGE gels and electroblotted to PVDF paper. The

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Abbreviations: IPTG, isopropyl thiogalactoside; PVDF, poly(vinylidene difluoride); BSA, bovine serum albumin.

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blots were soaked in blocking buffer (0.5% Tween/0.5 M NaCl/10 mM Tris, pH 8.2) containing 2.5% bovine serum albumin (BSA) for >1 h. The blots were then incubated sequentially for 1 h each in blocking buffer containing the indicated concentrations of periplasmic extract or purified proteins (see figure legends), anti-serum against appropriate Pap proteins, and finally anti-serum against rabbit IgG conjugated to either alkaline phosphatase or horseradish peroxidase. Between successive incubations, the blots were rinsed three times with blocking buffer. Blots were developed by using 50 μg of 5-bromo-4-chloro-3-indolyl phosphate per ml and 0.01% nitrotetrazolium blue in developing buffer (3 mM $MgCl_2$/50 mM Tris, pH 9.8) (see Fig. 3) or by using an enhanced chemiluminescent (ECL) detection kit (Amersham) and autoradiography (see Fig. 2C). Densitometry of the autoradiograms was done with an LKB Ultroscan densitometer.

PapC Plate Binding Assay. Fifty microliters of membrane extracts from HB101/pKD101 or HB101 (as a control) in phosphate-buffered saline; (PBS; 120 mM NaCl/2.7 mM KCl/10 mM phosphate buffer salts, pH 7.4; Sigma) was allowed to adhere to microtiter wells overnight at 4°C. After blocking the wells for >1 h with 3% BSA in PBS, wells were sequentially incubated for 45 min each with the indicated amounts of periplasmic extracts or purified proteins (see Fig. 2 legend), followed by anti-PapD antiserum, and finally anti-rabbit IgG conjugated to alkaline phosphatase. Between successive incubations the wells were rinsed four times with PBS. The assays were read on a Molecular Devices (Menlow Park, CA) thermo-max microplate reader after incubation with 1 mg of *p*-nitrophenyl phosphate per ml in 10 mM diethanolamine/1 mM $MgCl_2$, pH 9.5.

RESULTS

Overproduction and Partial Purification of PapC. The production of PapC was examined after induction with 0, 0.1, 1.0, or 10.0 mM IPTG by analyzing outer membrane extracts by SDS/PAGE (Fig. 1A). A protein migrating with the predicted molecular mass of PapC (86 kDa) was found in induced cultures (Fig. 1A, lanes 2–4) but not in uninduced cultures (Fig. 1A, lane 1). The 86-kDa protein present in induced HB101/pKD101 outer membrane extracts was partially purified and shown by amino-terminal sequencing to be PapC (Fig. 1B and C; ref. 12). In addition to the 86-kDa band, a major band at ≈70 kDa was also present in outer membranes of induced cultures but not in uninduced cultures (Fig. 1A). The amino-terminal sequence of this protein was identical to that of full-length PapC, identifying the 70-kDa protein as a large amino-terminal truncate of PapC.

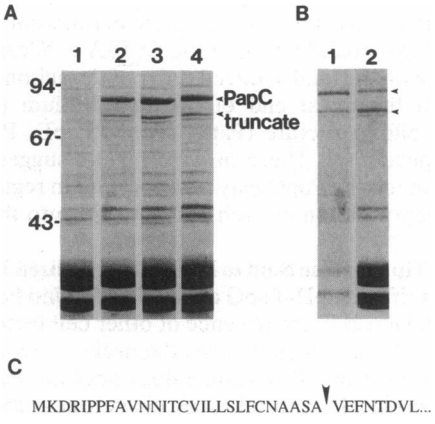

FIG. 1. Overproduction and partial purification of PapC. (A) Outer-membrane proteins from HB101 carrying pKD101 grown in 0.0 (lane 1), 0.1 (lane 2), 1.0 (lane 3), and 10.0 mM IPTG (lane 4) were electrophoresed through a SDS/10% PAGE gel and stained with Coomassie blue. PapC and a truncate of PapC (denoted by arrowheads) increased with increasing IPTG concentration. (B) A Coomassie blue-stained SDS/PAGE of proteins in the supernatant (lane 1) and pellet (lane 2) of outer membranes containing PapC after freeze/thaw treatment is shown. The supernatant contained a substantial amount of PapC, while the majority of the other outer-membrane proteins were pelleted. (C) The PapC and PapC truncate bands (denoted by arrowheads in B) were electroblotted to PVDF paper and isolated, and their amino-terminal sequence was determined. The sequence of prePapC is shown. The full-length PapC and the PapC truncate had identical amino-terminal sequences starting with the valine at position 29 (indicated by the arrow) of the prePapC sequence.

Unlike what has been reported for other members of the PapC family (19), there appeared to be no loss of viability in cells that overproduced PapC. The wet weight of cells recovered from uninduced or induced cultures of HB101/pKD101 (*papC*) or HB101 harboring the vector alone (HB101/pMMB91) was approximately the same (data not shown).

Chaperone–Subunit Complexes Bind Differentially to PapC. The mechanism by which chaperone–subunit complexes are targeted to outer membrane assembly sites was investigated in an *in vitro* ELISA assay in which various concentrations of native purified PapD, PapD–PapG, PapD–PapK, and PapD–PapA complexes were tested for their ability to bind PapC-coated microtiter wells. Binding of the complexes to PapC was detected and quantitated by using anti-PapD antiserum (Fig. 2A). PapD alone did not bind significantly to PapC. In contrast, purified PapD–PapG complexes bound strongly to PapC (Fig. 2A). Remarkably, purified PapD–PapA and PapD–PapK showed no specific binding to PapC even at concentrations 10-fold higher (10 μg/ml) than those at which PapD–PapG showed specific binding (1 μg/ml). These *in vitro* results suggest an effector function for PapD *in vivo*, where it would only be targeted to PapC after binding certain subunit types.

Since PapD–PapE and PapD–PapF complexes have not yet been purified, their ability to bind to PapC was assessed in a similar ELISA experiment by using various dilutions of crude periplasmic extracts containing the various complexes. Periplasmic extracts were obtained from strains in which PapD was coexpressed with PapA [HB101/pPAP43(*papIBA*; ref. 20) + pLS101(*papD*; ref. 21)], PapE [HB101/pPAP63(*papE*; ref. 10) + pLS101(*papD*)], PapF [HB101/pFJ7(*papF*; ref. 7) + pLS101(*papD*)], PapK [HB101/pFJ11(*papK*; ref. 7) + pLS101(*papD*)], or PapG [HB101/pJP1(*papDG*; C. H. Jones, J. Pinkner, S. N. Abraham, and S.J.H., unpublished data)] (Table 1). The concentration of each subunit was determined by densitometry of SDS/PAGE gels, and each binding curve was normalized as described in the legend to Fig. 2. Fig. 2B shows that, as expected from the purified protein assays, PapD–PapG complexes had the strongest binding to PapC, while both PapD–PapK and PapD–PapA did not bind. Periplasmic PapD–PapF complexes bound PapC at an intermediate level between that of PapD–PapK and that of PapD–PapG. PapD–PapE complexes bound slightly better than either PapD–PapK or PapD–PapA. These results showed differential abilities of the various chaperone–subunit complexes to bind to PapC *in vitro* that paralleled their order of incorporation into the growing pili *in vivo*.

Targeting of Periplasmic Complexes to PapC. Immunoblot (Western) analyses were done to investigate whether PapC and the ≈70-kDa PapC truncate were specifically recognized by the chaperone–subunit complexes (Fig. 3). PVDF-bound PapC was incubated with periplasmic extracts from HB101, HB101/pLS101(*papD*; ref. 21), and HB101/pPAP58(*papDJKEFG*; ref. 9). The binding of PapD or PapD-subunit complexes to PapC was then detected in immunoblots by using anti-PapD antiserum. PapD did not bind the immobilized PapC

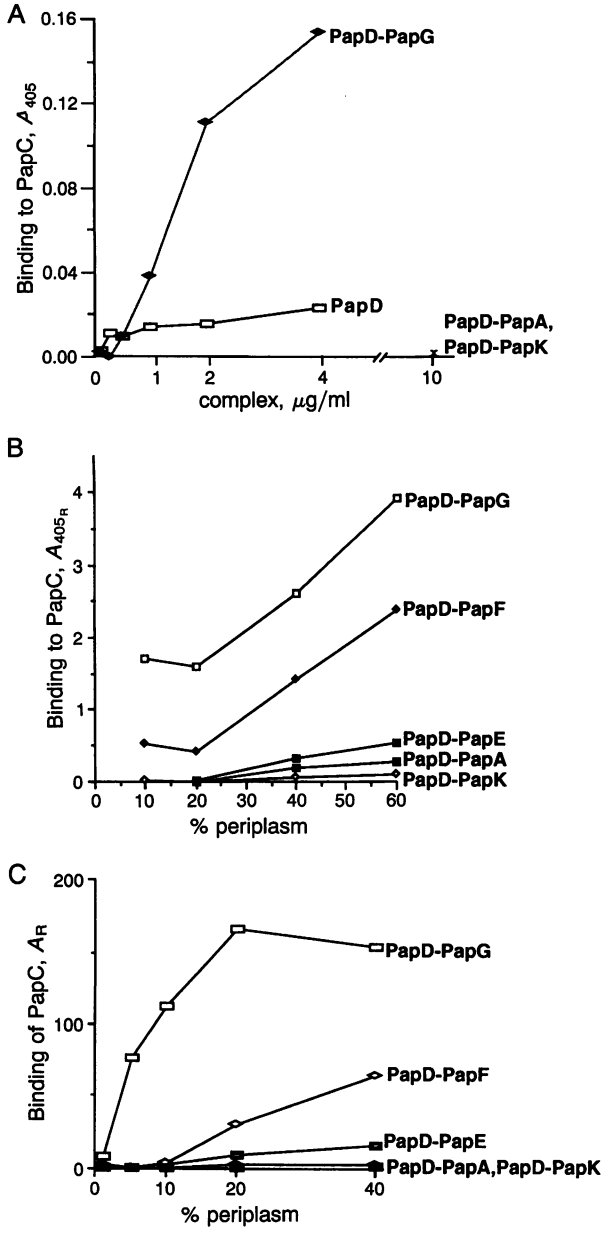

FIG. 2. Chaperone preassembly complexes bind to PapC. Partially purified PapC was allowed to adhere to microtiter wells (*A* and *B*) and then treated with 0.125, 0.25, 0.5, 1.0, 2.0, or 4.0 μg of PapD or PapD–PapG per ml or 10 μg of PapD–PapK or PapD–PapA per ml in PBS containing 3% BSA (*A*). PapC wells were also treated with 10%, 20%, 40%, or 60% periplasmic extracts in PBS containing 3% BSA (*B*). Binding of either purified proteins or periplasmic components was quantified by using anti-PapD and anti-rabbit IgG alkaline phosphate conjugate. In *A* and *B*, binding to PapC was determined as the difference between the average absorbances at 405 nm of wells coated with partially purified PapC and those coated with identically prepared HB101 membrane supernatants. (*C*) Binding of periplasmic extracts to PVDF-immobilized PapC was performed and quantitated by densitometry as described in text. Periplasmic extracts were diluted and used at 1%, 5%, 10%, 20%, and 40%. Binding to PapC was determined as the sum of the absorbance intensities of the PapC and ≈70-kDa PapC truncate bands due to the binding of chaperone–subunit complexes. The concentrations of the Pap subunit proteins in the different periplasmic extracts used in *B* and *C* were quantitated by using densitometry of Coomassie blue-stained SDS/PAGE and the known concentration of the added lysozyme band. PapK, PapE, PapA, PapG, and PapF were found to be at ≈300, 120, 115, 100, and 50 μg/ml in their respective periplasmic extracts. To compensate for the relative subunit concentrations, $[subunit]_R$, the raw absorbance binding data for a particular chaperone–subunit complex was multiplied by the relevant subunit concentration as compared with that of the most abundant subunit, PapK—[PapK]/[relevant subunit] = $[subunit]_R$—to give A_{405_R} for microtiter well assays (*B*) and A_R for quantitative western assays (*C*).

Table 1. Characteristics of plasmids

Plasmid	Relevant genotype	Ref.
pLS101	*papD*	21
pPAP43	*papA*	20
pPAP58	*papDJKEFG*	9
pPAP63	*papE*	10
pFJ7	*papF*	7
PFJ11	*papK*	7
pJP1	*papDG*	*
pKD101	*papC*	This study

*C. H. Jones, J. Pinkner, S. Abraham, and S.J.H., unpublished data.

(Fig. 3*A*, lane 2). However, both the 86-kDa full-length PapC band and the 70-kDa amino-terminal truncate of PapC were specifically recognized by PapD-subunit complexes (Fig. 3*A*, lane 3).

To confirm that both components of the chaperone–subunit complexes were present in the species recognizing PapC, anti-tip fibrillum antiserum, which recognized PapG, PapF, PapE, and PapK, was also used to detect binding. Fig. 3*B* shows that anti-tip fibrillum antiserum detected the binding of the periplasmic PapD–PapG, PapD–PapE, and PapD–PapF complexes to PapC (Fig. 3*B*, lanes 2–4). Interestingly, PapD–PapK complexes did not bind to PapC in amounts that could be detected with either anti-tip fibrillum antiserum (Fig. 3*B*, lane 5) or anti-PapD antiserum (data not shown). Similarly, PapD–PapA complexes did not bind to PapC in amounts that could be detected with either anti-pilus (Fig. 3*C*, lane 1) or anti-PapD antiserum (Fig. 3*C*, lane 2).

The binding of various dilutions of chaperone–subunit complexes to PVDF-bound PapC was also developed by using a chemiluminescent substrate together with the same anti-PapD antiserum. As seen in Fig. 2*C*, the relative differences in the ability of each chaperone–subunit complex to bind PapC were the same in this quantitative Western assay as in the ELISA assays (Fig. 2 *A* and *B*). Interestingly, the relative affinities of the respective chaperone–subunit complexes for PapC found by either the ELISA or Western assay correlated with the final deduced order of the subunits as they occur from the distal end of the tip fibrillum (7) in the composite pilus structure (PapG, PapF, PapE, PapK, and PapA, respectively). These *in vitro* results suggest that interactions involving PapC may be important in regulating the order of incorporation of each subunit type into the pilus *in vivo*.

Purified Tip Fibrillae Bind to PVDF-Immobilized PapC. The ability of purified PapD–PapG complexes to bind PapC (Figs. 2*A* and 3*D*, lane 2) in the absence of other cell factors shows that the PapD–subunit complexes themselves recognize specific motifs on PapC. PapD alone does not bind PapC (Figs. 2*A* and 3*D*, lane 1), and different chaperone–subunit complexes differentially bind to PapC, suggesting that the subunits and not the chaperone contain the interactive surfaces recognized by PapC. Therefore, purified tip fibrillae were tested for their ability to bind PapC in a Western blot. Lane 4 in Fig. 3*D* shows that tip fibrillae composed of PapG, PapF, PapE, and PapK (7) were able to bind to PapC in the absence of PapD as detected with anti-pilus antiserum. Although this does not exclude a direct interaction between PapD and PapC when a preassembly complex binds to PapC, it argues that the subunit component of the complex contains interactive motifs sufficient to recognize PapC. *In vivo*, PapD is required for subunit stability and may influence subunit binding to PapC

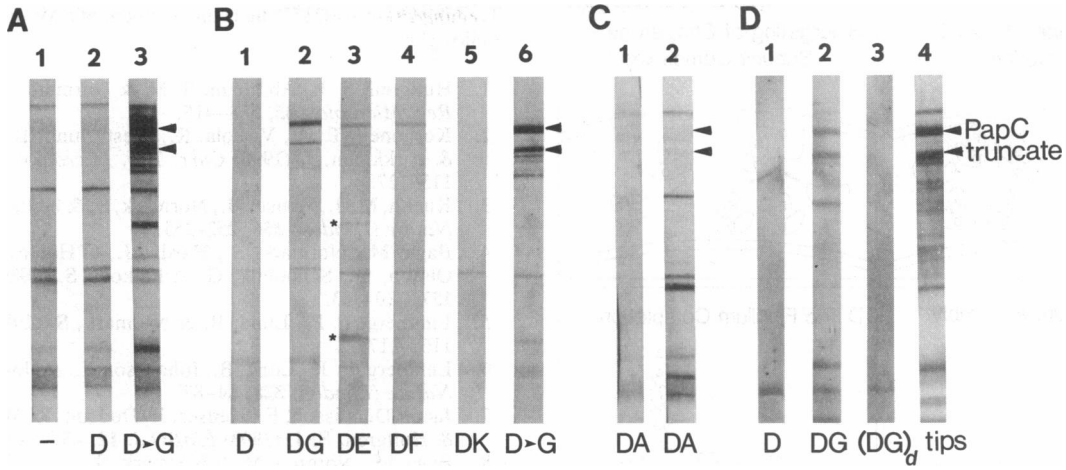

FIG. 3. Chaperone preassembly complexes bind to PapC immobilized on PVDF. Partially purified PapC was transferred to PVDF after SDS/PAGE and treated with periplasmic extracts as described in text. The positions of PapC and the PapC truncate are indicated by arrowheads. (*A*) Periplasmic extracts (20%) containing the Pap proteins indicated were allowed to bind to PVDF-bound PapC. Binding of PapD complexes was detected by immunoblotting with anti-PapD antiserum. (*B*) PVDF-bound PapC was treated with 20% periplasmic extracts containing the Pap proteins indicated. Binding of Pap proteins in this experiment was detected by immunoblotting with anti-tip fibrillae antiserum. (*C*) PVDF-bound PapC was treated with 20% periplasmic extracts containing PapD–PapA followed by immunoblotting with anti-pilus (lane 1) or anti-PapD (lane 2). No binding of PapD–PapA was detected. (*D*) PVDF-bound PapC was treated with 1 μg of purified PapD per ml (lane 1), 1 μg of purified PapD–PapG per ml (lane 2), 1 μg of heat-denatured PapD–PapG per ml (lane 3), or purified tip fibrillae (lane 4). Binding of the added proteins to PapC was detected by immunoblotting with anti-PapD–PapG (lanes 1–3) or anti-pilus antisera (lane 4). Staining of membrane proteins unrelated to PapC was due to nonspecific binding of the antisera (data not shown) or binding of PapD–PapE to host membrane proteins (as indicated by asterisks in lane 3 of *B*).

by promoting the proper subunit tertiary structure recognized by PapC.

To investigate the requirement for the proper conformation of the subunits in binding PapC, we tested the ability of heat-denatured PapD–PapG complexes to bind PapC. PapG has been found to exist in a native-like conformation when bound to PapD (1, 9, 11, 22). When purified PapD–PapG was first denatured by heating to 95°C before incubation with PVDF-bound PapC, no binding to PapC was detected (Fig. 3*D*, lane 3) as determined by immunoblotting with anti-PapD–PapG antiserum. These results show that the binding of PapD–PapG to PapC in this *in vitro* assay is dependent on a heat-sensitive conformation.

DISCUSSION

Pilus biogenesis involves the conversion of the chaperone–subunit complexes into supramolecular adhesive surface fibers having a distinct architecture. This conversion process depends on PapC, since it does not occur in PapC's absence (12). Our *in vitro* studies have suggested that PapC might regulate the ordered targeting of chaperone–subunit complexes to the outer membrane assembly site where the chaperone is dissociated from the respective subunits, allowing their polymerization into pili.

In this study, fully and partially purified chaperone and chaperone–subunit complexes and partially purified PapC were used in two different *in vitro* assays to investigate and quantitate complementary protein interactions between PapC and chaperone–subunit complexes. The complexes that PapD forms with each of the three most distal tip fibrillum subunits (PapG, PapF, and PapE) were able to bind to PapC in either of our assay systems. Interactions between these complexes and PapC *in vivo* could direct their targeting to the outer membrane assembly site. Interestingly, the complexes between PapD and the most proximal subunit of the tip fibrillum (PapK) and the major rod subunit (PapA) did not bind to PapC. The ability of PapD–PapG, PapD–PapF, and PapD–PapE and the inability of PapD–PapA and PapD–PapK to bind to PapC suggest a mechanism by which pilus tips are made before pilus rods. This would ensure that every pilus rod is joined end-to-end to an adhesive tip fibrillum.

The differential affinities of the various pilus proteins for PapC and PapD, the relative abundance of each of the subunit proteins and the complementary surfaces on each subunit type all appear to be factors that influence ordered assembly. These factors argue that the cascade of protein–protein interactions that leads to the formation of an adhesive composite pilus fiber proceeds according to a model similar to the one proposed in Fig. 4. PapD binds to each subunit as it is translocated into the periplasmic space (Fig. 4*A*). The resulting preassembly complexes are then targeted to PapC. In the initial stage, PapD–PapG has the highest relative affinity for PapC and thus binds to PapC first (Fig. 4*B*). The subsequent binding of PapD–PapF to PapC is thought to trigger chaperone uncapping and initiate tip fibrillum growth (7). In addition, PapF has the only complementary surfaces capable of correctly linking PapG to PapE (7). PapE subunits are then able to polymerize into a tip fiber upon multiple rounds of PapD–PapE binding, uncapping, and PapE incorporation (Fig. 4*C*). The inability of PapD–PapK and PapD–PapA to efficiently bind to empty PapC sites would ensure that pilus rods are not made in the absence of tip fibrillae. We propose that the binding site for PapD–PapK may be the polymerized tip fibrillum in the context of PapC. Incorporation of PapK is known to terminate the growth of the tip fibrillum and, we also propose, would create a binding site for PapD–PapA (Fig. 4*D*). This hypothesis is supported by the discovery that PapK has been shown to be the only tip protein that, when expressed by itself, is able to nucleate the formation of a pilus rod (7). We propose that, upon chaperone uncapping and PapK incorporation into the growing fiber, tip growth is terminated and the targeting of PapD–PapA complexes to the assembly site is initiated, allowing polymerization of pilus rods (Fig. 4 *E* and *F*).

Previously it was suggested that PapC was a porin-like molecule (12), forming a channel in the membrane through which pilus subunits were able to pass. This study proposes that if PapC forms a pore for the pilus, it is not passive but instead has an active role in the determination of the order of pilus subunit passage. We refer to this ordering function as

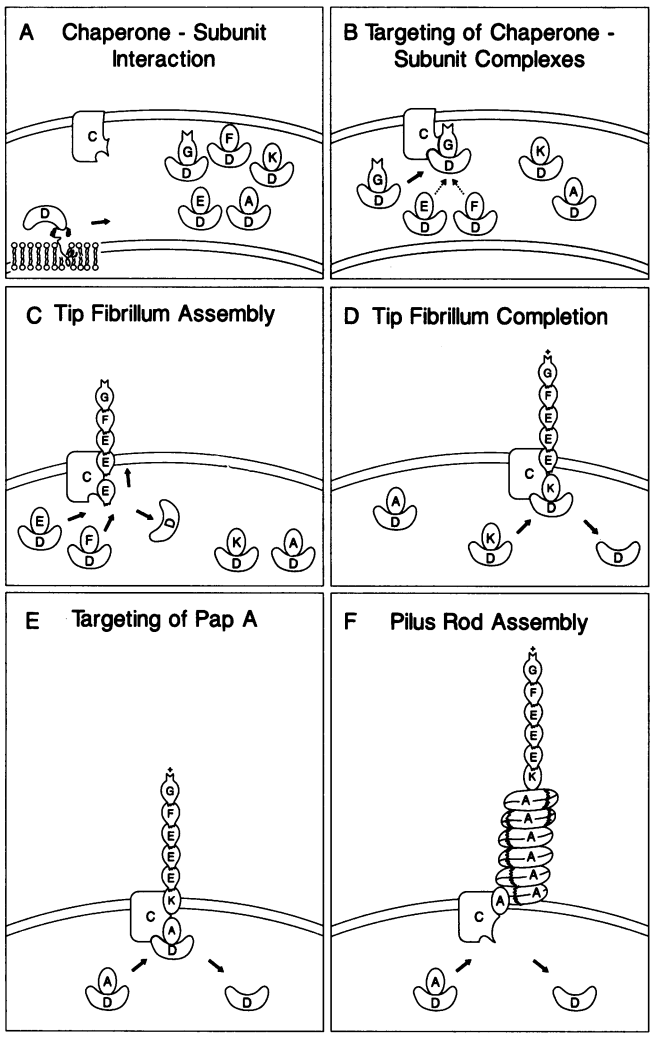

FIG. 4. Model of P pilus biogenesis involves the ordered targeting of preassembly complexes to PapC (see text for details).

that of an "usher," since, like a human usher, we suggest that PapC is able to distinguish between "ticket holders," allowing them entry only at the proper time and place. PapC is a representative member of a family of outer membrane ushers, including FanD, FaeD, FimD, MrkC, CaflA, FhaA, F17c, C53–104-kDa, and an 84-kDa *Salmonella* protein (19, 23–26, 28–32), which are required for pilus assembly in Gram-negative bacteria. It is likely that all of these proteins have similar ushering functions acting in concert with their respective chaperone partners (27) to assure the correct interactions necessary for the production of ordered adhesive structures, which are important in colonization and infection of susceptible hosts.

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