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MINIREVIEW

Adhesin Presentation in Bacteria Requires Molecular Chaperones and Ushers

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INTRODUCTION

Bacteria have developed complex and varied mechanisms to present adhesins to eukaryotic receptors which promote attachment and colonization of mucosal surfaces and in many cases the subsequent invasion of these tissues (4, 32, 39). A key event in colonization is surviving the mechanical cleansing of the mucosal surface. Most pathogens avoid elimination by bulk flow through high-affinity attachment to specific cell surface glycolipid and glycoprotein architectures (30, 39). Long, hairlike extracellular appendages called pili, produced by most gram-negative pathogens, mediate specific attachment to the epithelial cell surface (16, 18). Often associated with the pilus is a protein called an adhesin, which directs high-affinity binding to specific cell surface components (1, 24). For example, uropathogenic strains of *Escherichia coli* expressing P pili overcome initial barriers to infection by expressing an adhesin molecule which specifically binds to the α-D-galactopyranosyl-(1-4)-β-D galactopyranoside [Galα(1-4)Gal] moiety present in the globoseries of glycolipids on cells lining the urinary tract (24). This bacterial attachment event is the result of a stereochemical fit between an adhesin located at the pilus tip and specific receptor architectures on uroepithelial cells. The intention of this review is to describe general mechanisms used by gram-negative bacteria to present adhesins in configurations which make them able to recognize receptors on the surface of epithelial cells. We will focus on P pilus biogenesis as a prototype system to investigate how protein subunits fold into domains that serve as modules for building up large surface assemblies required for bacterial attachment.

DIGALACTOSIDE BINDING ADHESIN MOLECULES

Lund et al. (27) demonstrated that the gene product of the *papG* locus contained the disaccharide-specific binding activity of the P pilus. Deletion of the *papG* structural gene had no effect on pilus formation; however, the pili isolated from a *papG* deletion strain were not adhesive. Transcomplementation of a *papG* deletion with a gene encoding a related adhesin, PrsG, changed the binding specificity of the pilus from human erythrocytes to sheep erythrocytes, definitively assigning the role of adhesin to PapG (27).

Tissue and host tropisms of uropathogenic *E. coli* isolates seem to depend on the binding specificity of the microbe for digalactoside-containing isoreceptors located on the uroepithelium. The saccharide portion of the glycolipid isoreceptor, which contains the Galα(1-4)Gal moiety, is structurally diverse and anchored in the membrane by a ceramide group (12). Stromberg et al. (34) examined the receptor binding specificity of four allelic variants of PapG and compared them with those of clinical isolates of *E. coli* associated with urinary tract infections from human and dog. Their studies revealed three different epitopic binding variants for Galα(1-4)Gal-containing isoreceptors. All four G adhesins mediated binding to the digalactoside-containing glycolipids; however, differences in binding due to neighboring sugar groups present proximal to the disaccharide in the isoreceptor were detected. The important revelation coming from these studies was that the host species from which the wild-type uropathogenic strains were isolated seemed to determine the isoreceptor recognition specificity. In addition, these investigations revealed that the restriction of specific isoreceptors to cell types, tissues, and species resulted in the apparent tissue and host tropisms of various isolates of pyelonephritis-causing strains of *E. coli*. Recently, Stromberg et al. (35) showed for the first time that the orientation of the saccharide portion of each isoreceptor, in relation to the membrane, determined the binding specificity of the four allelic PapG adhesin variants. The addition of saccharide residues proximal to the Galα(1-4)Gal residue resulted in presentation of different receptor architectures that were restrictive for certain allelic PapG adhesins. These important studies were the first to explain how variation in PapG adhesins among wild-type strains has provided for optimal interaction with digalactoside-containing isoreceptors resulting in the observed host and tissue tropisms.

A STRATEGY FOR ADHESIN PRESENTATION

The importance and implied specificity of the binding event suggest that the adhesin molecule must be exposed or "presented" in an active binding conformation apart from the interfering molecular structures and negatively charged molecules present on the surface of *E. coli*. Adhesive P pili are virulence determinants associated with pyelonephritis-causing strains of *E. coli* (16, 18). The adhesin, PapG, is part of a specialized fibrillar structure found at the tip of a pilus rod (Fig. 1) (20). The shaft of the pilus is composed of repeating monomers of the major pilin subunit PapA and is approximately 5 to 7 nm in diameter. The adhesive tip structure, called the tip fibrillum, is composed mainly of repeating PapE subunits and is joined end to end to the pilus rod. The diameter of this unique structure is approximately one-third that of the shaft. PapG, the Galα(1-4)Gal adhesin, is localized at the distal end of the fibrillum (20). Two minor

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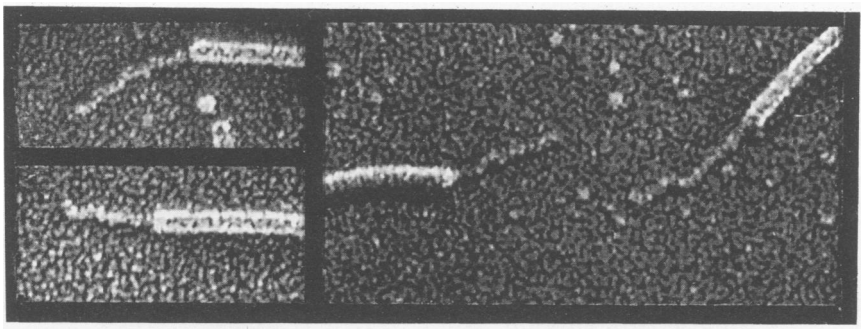

FIG. 1. Freeze-etch electron microscopy demonstrates the flexible fibrillum tip of purified P pili. Reprinted from *Nature (London)* (20) with permission of the publisher.

pilins, PapF and PapK, are also found in the tip fibrillum and seem to play a role in regulation of tip length and linking of the adhesin moiety to the fibrillum (19). The composite architecture of the P pilus fiber reveals the strategy used by uropathogenic *E. coli* to present the PapG adhesin to eukaryotic receptors. The rigid PapA rod extends the adhesin away from interference by lipopolysaccharide and other components at the bacterial cell surface, while the flexible fibrillum allows PapG steric freedom to recognize and bind to the digalactoside moiety on the uroepithelium.

Two proteins, PapC and PapD, encoded in the *Pap* operon, are required for the production of adhesive pili but are not a part of the final structure. PapD has been defined as a periplasmic chaperone (15, 21, 26), a protein required for the folding or assembly of another protein (9, 10). In the absence of PapD, the pilus subunit proteins are proteolytically degraded. In the absence of PapC, unassembled pilus proteins accumulate in the periplasm (29). Recent studies have identified a novel activity for PapC (8). This large membrane-associated protein receives the various pilus subunits delivered by the chaperone and ushers them into the pilus in a defined order. In accordance with its activity, we have named PapC a molecular usher (8). The analogy follows that the chaperone prevents inappropriate subunit interactions, while the usher allows correct subunit interactions to occur and regulates the order in which the subunits are placed in the pilus by making the appropriate "introductions."

The P pilus system is ideal for studying the general principles in which interactive monomeric subunits are delivered across a cytoplasmic membrane, transported through the periplasm, and, finally, assembled across the outer membrane. The pilus subunit proteins travel through three postcytoplasmic cellular compartments, and their folding and assembly seem to be controlled at each step of the trip by an accessory protein. The well-understood genetics of the *pap* system have allowed extensive manipulation of the various components of the pilus system and provided for an initial assignment of function to most of the gene products (26, 30) (Fig. 2). The mechanisms which regulate the correct protein-protein interactions required for ordered assembly of composite adhesive pilus fibers are discussed below.

ROLE OF A PERIPLASMIC CHAPERONE IN PILUS BIOGENESIS

Each P pilus is composed of approximately 1,000 proteins of six different types (16). With 200 to 300 pili per bacterium, a system must be in place to prevent premature nonproductive interactions of the $\sim 3 \times 10^5$ different subunit types prior to their delivery to outer membrane assembly sites. Moreover, because of the distinct heteropolymeric architecture of the pilus, PapA monomers must be added into the growing pilus only after the adhesive fibrillar tip has been formed. The mechanisms which regulate the correct protein-protein interactions required for formation of distinct composite pilus fibers have been studied in vitro. PapD forms periplasmic complexes with the pilus protein protomers. The PapD-PapG (DG) complex has been purified from the periplasm by Galα(1-4)Gal affinity chromatography and extensively characterized in vitro (17, 21). PapD and PapG exist in a 1:1 molar ratio in the purified periplasmic complex. The ability to purify the complex by utilizing the binding specificity of PapG for its receptor suggested that the adhesin is in a native-like conformation when bound to PapD. In contrast, cytoplasmic chaperones seem to bind proteins in a nonspecific manner and maintain them in an unfolded state (13, 22, 23, 36). Binding of PapD to PapG is reversible, as would be expected since PapG must be released into the growing pilus. Interestingly, the release mechanism is seemingly ATP

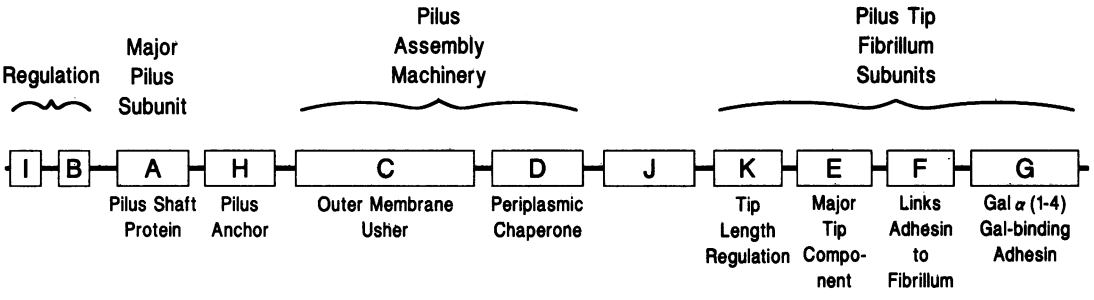

FIG. 2. The *pap* operon and the assigned role in genetic regulation and pilus biogenesis of 10 of the 11 gene products.

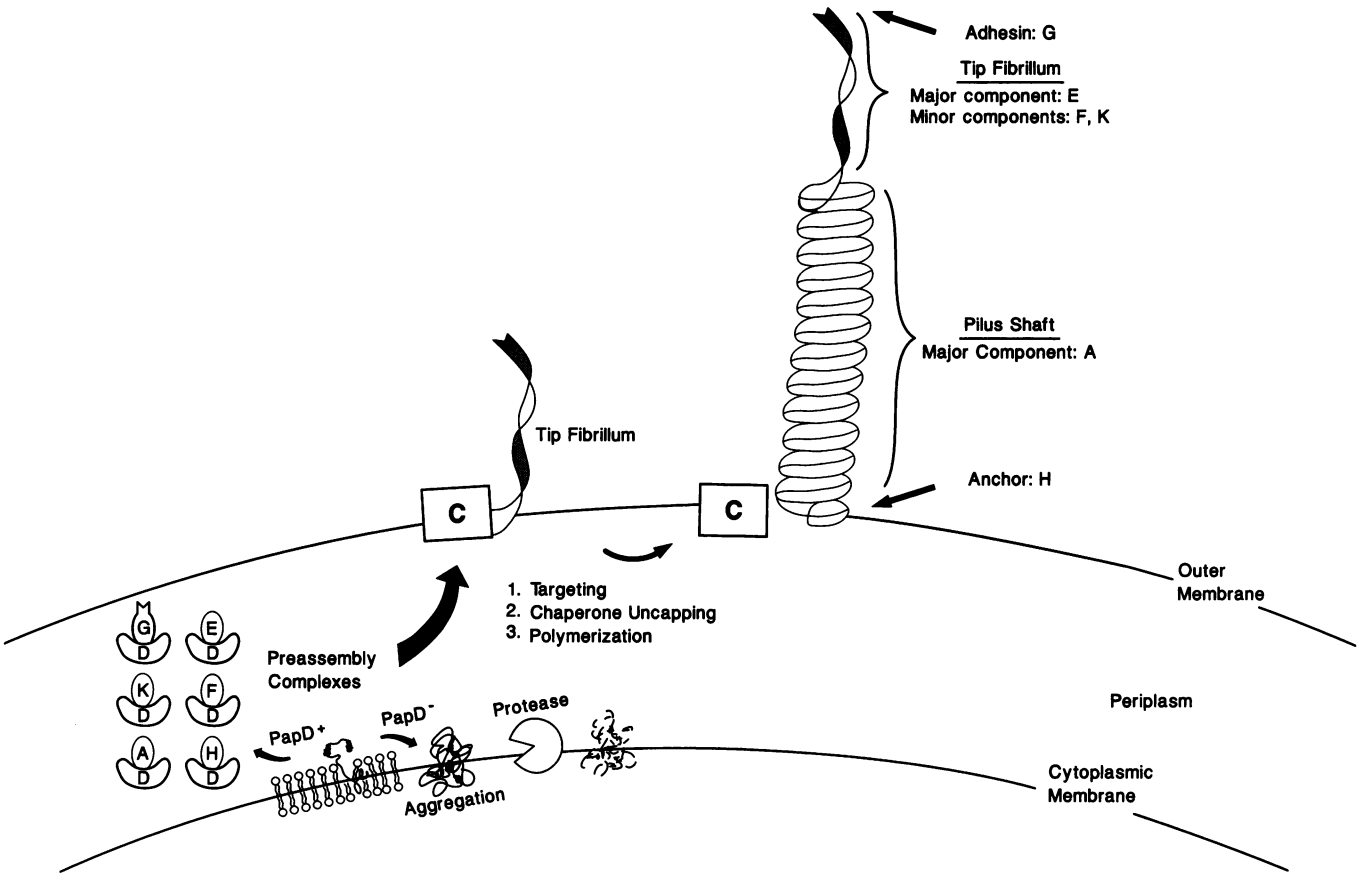

FIG. 3. Model of pilus subunit transport through the periplasm and pilus assembly. In the absence of PapD, nascent subunits aggregate and are subjected to proteolytic degradation. Association with PapD to form preassembly complexes caps interactive surfaces, preventing aggregation, and stabilizes the subunits. The bimolecular complexes are targeted to the membrane assembly site, PapC (C), where uncapping of PapD occurs and pilus assembly is directed. As a result of the differing affinities of the chaperone subunit complexes for PapC (C), the adhesin resides at the terminus of the fibrillum, which is joined end to end to the pilus rod.

independent (16). In vivo, release of PapD from the DG complex may be facilitated by the interaction of DG with an outer membrane assembly site, as discussed below. The DG complex can be dissociated in vitro under reducing conditions in the presence of 4 M urea. Dilution of the denaturant, however, fails to allow reformation of the complex; instead, the proteins aggregate. Remarkably, the presence of native PapD in the diluent allows the DG complex to reform (21). Apparently, exposure of interactive surfaces leads to subunit aggregation, which blocks folding. The ability of PapD to bind and cap interactive surfaces on the subunit presumably allows proper folding by blocking nonproductive interactions. This in vitro activity may reflect the in vivo role of PapD to bind to newly translocated unfolded proteins and maintain them in assembly-competent conformations.

In vivo, we envision two competing pathways for each interactive monomeric subunit as it crosses the cytoplasmic membrane into the periplasm (Fig. 3). In one pathway, interactive surfaces of a pilus subunit protein, destined to interact with other subunits in the final pilus structure, drive the formation of insoluble aggregates because of premature and inappropriate interactions in the periplasmic space. The premature association of these interactive surfaces probably prevents proper folding and thus targets the aggregated subunits to proteolytic degradation pathways. The PapD chaperone physically caps or covers the interactive surfaces on the subunits by directly binding to them as they emerge from the cytoplasmic membrane forming periplasmic preassembly complexes (21). The subunits are then able to properly fold into domains that serve as modules for building up the ordered pilus architecture.

PapD CHAPERONE FAMILY

PapD is the prototype member of a large family of periplasmic chaperones. In most pilus systems analyzed thus far, a periplasmic protein with homology to PapD has been identified (15). In all cases, this protein is required for pilus expression but is not a part of the final structure.

The three-dimensional structure of PapD has been solved to a resolution of 2.5 Å by Holmgren and Branden (14) and recently to a resolution of 2.0 Å by Ogg and Branden (31). PapD consists of two globular domains oriented toward one another, with the overall shape similar to that of a boomerang (Fig. 4). Each domain is a β-barrel structure formed by two antiparallel β-pleated sheets and has a topology similar

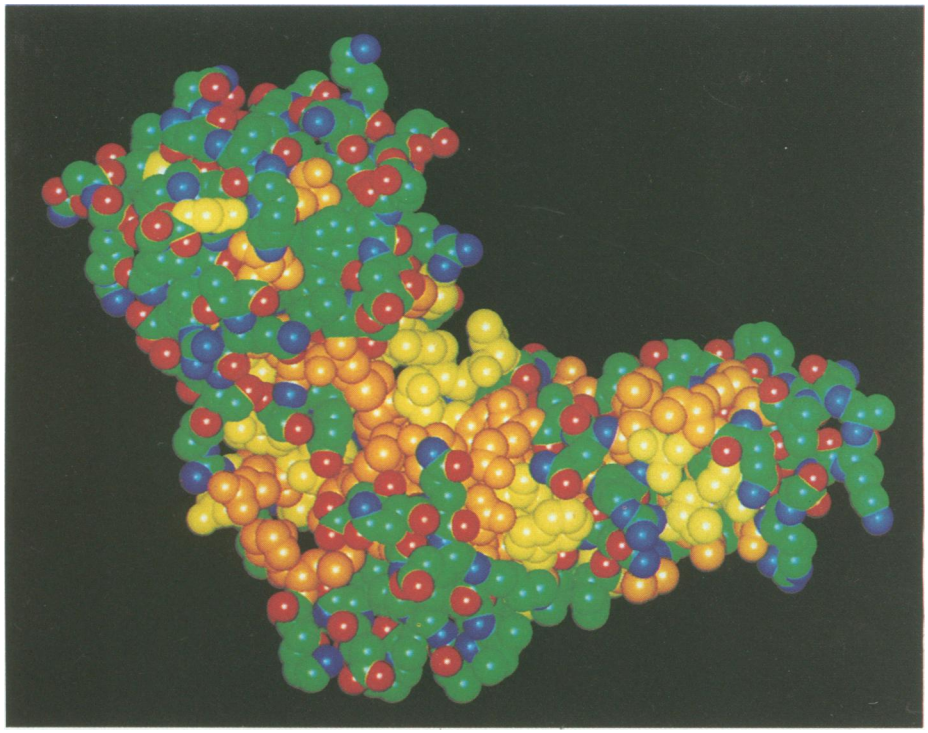

FIG. 4. A space-filling model of PapD oriented such that the viewer is facing into the putative subunit binding pocket. Invariant residues in this family of proteins are shown in yellow, while conserved residues are shown in orange. All other residues are colored by atom such that carbon, nitrogen, and oxygen are green, blue, and red, respectively.

to that of an immunoglobulin (Ig) fold. The proposed subunit binding pocket of the PapD chaperone is located in the cleft of the molecule, which is formed between the two β-barrel domains (14, 33).

The sequences of seven periplasmic pilus chaperones required for pilus assembly in *E. coli*, *Klebsiella pneumoniae*, and *Haemophilus influenzae* have been aligned and found to be 30 to 40% identical and 60% similar (15). A consensus sequence was derived and superimposed onto the crystal structure of PapD. This analysis revealed that most of the invariant and conserved residues in the chaperone family were critical to maintaining the structural integrity of the protein and were located in β-strands. In contrast, 70% of all loop residues are variable in this protein family, suggesting that amino acid residues in loop regions may be important in the specificity of chaperone-subunit interactions. Most invariant residues contributed to the hydrophobic core of the molecule. Three invariant residues were found to form an internal salt bridge necessary to orient the two domains toward each other to form the putative binding cleft. Another group of invariant residues were critical in positioning and orienting loop structures which link the β-strands (15).

Site-directed mutagenesis in invariant, conserved, and variable residues revealed that recognition of pilus subunit proteins by PapD involved the conserved cleft of the molecule (33). Slonim et al. (33) suggested that the PapD cleft contains specificity pockets which mediate interaction between PapD and pilus proteins. The proposed model suggests that PapD may differentially accommodate pilus subunit side chains in the cleft, resulting in different affinities between PapD and the pilus subunits. Furthermore, these differences in affinity may provide the precision required for assisting in the ordered biogenesis of an adhesive composite pilus.

MOLECULAR RECOGNITION EVENTS UTILIZING THE β-BARREL MOTIF: A COMMON THEME

The building block of the two domain PapD chaperone is an Ig-like β-barrel motif. This finding underlies the usefulness of this domain structure in protein recognition functions in bacteria as well as in higher-order organisms. In eukaryotes, both the Ig superfamily (2, 37, 38) (which includes antibodies, cell surface adhesion molecules, and T-cell receptors) and the cytokine receptor superfamily (3, 6) use the Ig fold for molecular recognition processes. The basic structure of this domain is best described as two antiparallel β-sheets packed tightly against each other to form a hydrophobic core (38). An Ig constant domain contains seven β-strands arranged in two β-sheets pinned together by a disulfide bond (Fig. 5a). All heavy and light chain constant domains have the same structure. An Ig variable domain contains two additional strands as well as a different order of the strands in the sheets, relative to a constant domain (5).

The Ig fold provides a stable platform for the display of specific recognition surfaces formed by either the loops connecting the β-strands or sequences located on the outer faces of the β-sheets (2, 25, 38). The recently reported structure of the human growth hormone receptor, $(hGHbp)_2$, complexed with its ligand has shown that it is a member of the cytokine receptor superfamily, which also makes use of the Ig fold for molecular recognition (7).

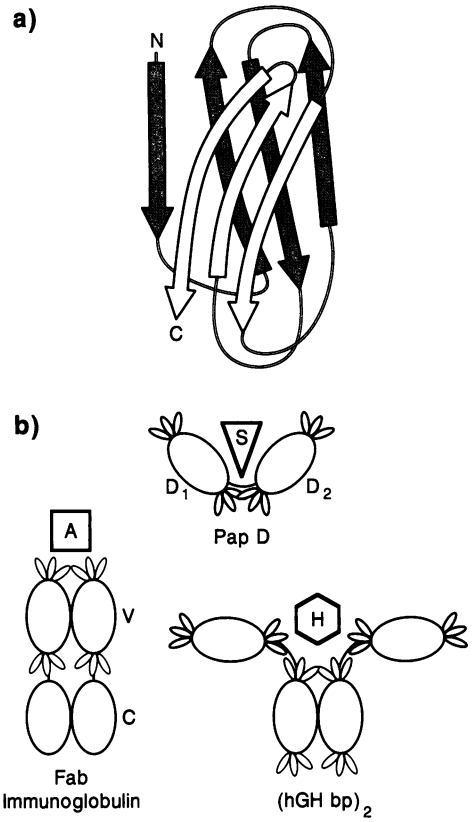

FIG. 5. (a) Schematic representation of the constant domain of an Ig molecule illustrating the β-barrel motif. The β strands in this two-sheet sandwich run antiparallel and are indicated by arrows, while the loops between the β strands are indicated by lines. (b) Protein binding strategies of three protein families utilizing the Ig fold: the pilus chaperone, PapD; the antigen-binding fragment of an Ig (Fab'); and the human growth hormone-binding protein $(hGHbp)_2$. The binding sites for antigen (A) and the human growth hormone (H) are well established, while the subunit binding pocket indicated for PapD is based on results of site-directed mutagenesis.

We suggest that in prokaryotes, the periplasmic pilus chaperone family has a structure which is a variation on the same theme. The second domain of PapD has structural features similar to the second domain of the human immunodeficiency virus receptor, CD4 (15). These domains differ from the classical constant domain organization by strand switching of one of the β-strands from the upper sheet to the lower sheet. PapD also lacks the intersheet disulfide bonds normally seen in constant domains (5). The first domain of PapD has a β-strand order similar to those of Ig variable regions (15). We hypothesize that this two-domain chaperone relies on a binding paradigm different from that used by antibodies or the growth hormone receptor, and this further corroborates the suggestion that these Ig-like domain-containing molecules make various uses of their surfaces to recognize ligand and partner proteins (Fig. 5b). Specifically, we suggest that PapD utilizes the Ig fold in two linked domains that are oriented such that a binding cleft is formed between the two domains. In this model, PapD binds subunits via side chain interactions with conserved residues in the cleft. Our current model suggests that the β-barrel structure also stabilizes variable loop regions that surround the cleft and that residues in the loops may impart specificity to the chaperone.

PILUS PRODUCTION REQUIRES AN OUTER MEMBRANE USHER PROTEIN

The final step in biogenesis of adhesive pili is incorporation of the subunits into a growing pilus in an ordered fashion. Ordered assembly restricts PapG to the tip of the fibrillum and regulates fibrillum length. Also, a single fibrillum is added end to end to each pilus shaft, and the length of the shaft is regulated. Obviously, production of a rod in the absence of a fibrillum or assembly of the pilus rod within the periplasm would be of no use to the bacterium. Dodson et al. (8) demonstrated that an outer membrane-associated protein, PapC, previously shown to be essential for P pilus assembly (29), plays a unique role in pilus biogenesis. This protein has been named a molecular usher. According to the American Heritage Dictionary (28), an usher is "(i) one who serves as an official doorkeeper. . ., (ii) a person employed to escort people to their seats. . ., or (iii) an official whose duty is to make introductions between unacquainted persons. . ." The name of a molecular usher is well suited for PapC since it functions in pilus biogenesis to direct an ordered progression of pilin subunits into the growing pilus as well as to direct "introductions" between subunit proteins (8).

PapC is a large protein of 88 kDa which appears to be conserved among all pilus-producing bacteria (8, 29). Among the genes that have been sequenced, the putative PapC homologs are 25% identical and 40% similar (8). Evidence suggests that the release of pilin subunits from the PapD bimolecular complex occurs in an ATP-independent step that depends on PapC (16). One model suggests that PapC "uncaps" PapD from the monomeric subunit, revealing the interactive surface on the subunit, and provides a structural platform for assembly of the subunits into the growing pilus by facilitating interactions with already assembled subunits. The well-choreographed assembly of the pilus may depend on the affinity of the subunit-PapD complexes for PapC as well as the relative concentration of each of the different bimolecular complexes present in the periplasm. Dodson et al. (8) found that the PapD-subunit preassembly complexes with the various pilin subunits are targeted to and bind to PapC with differing affinities. DG complexes bind to PapC with the highest apparent affinity, ensuring PapG's localization at the distal end of the pilus tip. PapD-PapE and PapD-PapF bind to PapC with an apparent affinity only slightly less than that of the DG complex, and PapF has been shown to be necessary to link PapG to the PapE fiber (19). In contrast, PapD-PapA complexes are targeted to PapC only when the usher is occupied by a growing tip fibrillum. This guarantees the presence of a tip fibrillum joined end to end to each pilus shaft. A simple model would suggest that PapC has one site for containment of the growing pilus and additional sites for interaction with incoming subunits presented in the context of the chaperone. While data suggest that the subunits, and not PapD, contain the interactive surfaces which recognize and bind to PapC, PapD is required for this interaction, presumably to maintain subunit conformation. When the conformation of the complex is destroyed in vitro or PapD is not present in vivo, the subunits are not able to be targeted to PapC and pilus assembly does not occur.

PERSPECTIVES AND DISCUSSION

In order for bacteria to withstand the mechanical cleansing and bulk flow associated with mucosal surfaces, specific

binding to surface architectures located on epithelial and mucosal surfaces must be achieved (16, 18). Epithelial cells lining the mucosal surfaces provide a myriad of surface glycolipids and glycoproteins, some of which pathogens specifically recognize via specific adhesin molecules (12, 30, 34, 35). Uropathogens expressing P pili preferentially bind to the globoseries of glycolipids containing the digalactoside Galα(1-4)Gal (24).

Interaction of pyelonephritis-causing strains of *E. coli* with cell surface isoreceptors is mediated through the P pilus composite fiber, which terminates in a flexible tip structure (20). The location of the adhesin in the tip fibrillum places it in an environment free of obstructing bacterial cell surface components and allows maximum flexibility for interaction with the receptor. P pilus tip fibrillae were discovered by utilizing a high-resolution freeze-etch electron microscopy technique (20) and may reveal a general structural feature that has yet to be described for other pili.

Investigating the role of chaperones and molecular ushers in pilus assembly has revealed several general biological principles which describe the pathway that monomeric subunits follow from synthesis to incorporation into extracellular organelles. When subunits cross into the periplasmic space as nascently translocated monomers, both productive and nonproductive pathways seem to be available. The productive pathway provides for proper subunit folding and targeting to pilus assembly sites, while the nonproductive pathway leads to aggregate formation followed by proteolytic degradation of the subunits. Molecular chaperones are essential in guiding subunits down biologically productive pathways (21). In gram-negative bacteria, periplasmic chaperones are probably of general importance in the expression of a wide array of surface structures which are composed of interactive subunits. This idea is supported by the discovery of the periplasmic chaperone family of proteins, which are required for the assembly of at least 10 different structures in five different organisms (15), and the recent identification of the caf1M protein in *Yersinia pestis* (11). caf1M is homologous to PapD and essential for the expression of the caf1 antigen of the *Y. pestis* capsule (11).

Another basic principle revealed in this pathway is that the chaperone must be displaced from the pilin subunit, a process we refer to as uncapping, to allow interactive surfaces to be exposed at the site of pilus polymerization (8, 21). The role of uncapping and directing subunit incorporation into the pilus is probably carried out by a membrane-associated molecular usher. In contrast to the chaperone's role in preventing inappropriate interactions in the periplasm, the molecular usher directs the appropriate "meeting" of subunits in both time and location (8).

In this review, we have described a strategy which gram-negative pathogens utilize to assemble and present adhesin molecules in an accessible location to allow for maximal interaction with cell surface-associated receptors. Adhesin presentation is essential for the critical first step in association with epithelial mucosa, which leads to colonization and further disease pathology.

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