Neisseria gonorrhoeae Type IV Pili Undergo Multisite, Hierarchical Modifications with Phosphoethanolamine and Phosphocholine Requiring an Enzyme Structurally Related to Lipopolysaccharide Phosphoethanolamine Transferases*[5]

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The zwitterionic phospho-forms phosphoethanolamine and phosphocholine are recognized as influential and important substituents of pathogen cell surfaces. PilE, the major pilin subunit protein of the type IV pilus (Tfp) colonization factor of Neisseria gonorrhoeae undergoes unique, post-translational modifications with these moieties. These phospho-form modifications have been shown to be O-linked alternately to a specific, conserved serine residue of PilE. However, the enzymes and precursors involved in their addition are unknown, and the full spectrum of PilE post-translational modifications has yet to be defined. Here, an intact protein-based mass spectrometric approach was integrated with bioinformatics and reverse genetics to address these matters. Specifically we show that a protein limited in its distribution to pathogenic Neisseria species and structurally related to enzymes implicated in phosphoethanolamine modification of lipopolysaccharide is necessary for PilE covalent modification with phosphoethanolamine and phosphocholine. These findings strongly suggest that protein phospho-form modification is mechanistically similar to processes underlying analogous modifications of prokaryotic sacccharolipid glycans. We also show that PilE undergoes multisite and hierarchical phospho-form modifications and that the stoichiometries of site occupancy can be influenced by PilE primary structure and the abundance of the pilin-like protein PilV. Together, these findings have important implications for the structure and antigenicity of PilE.

Covalent post-translational modifications of surface proteins in microbial pathogens are now a well established phenomenon. By modifying structure and potentially function, such post-translational modifications are likely to play an important role in the parasite-host interaction. Moreover, post-translational modifications provide effective means to augment the information content of compact genomes, to generate diversity, and to influence antigenicity. By way of example, covalent protein modifications with N- and O-linked carbohydrate appear more and more as common features of proteins of bacterial pathogens (1). Studies have revealed a surprisingly high degree of conservation and relatedness in a number of these systems, and in some instances glycosylation-defective mutants have been shown to be attenuated in virulence-associated properties (2, 3) and colonization (4–7). Nonetheless, the full significance of protein glycosylation has yet to be precisely defined in any prokaryotic system.

Type IV pili (Tfp)[8] are proteinaceous polymeric filaments that serve critical roles in disease pathogenesis and prokaryotic cell biology in many Gram-negative species. Important human pathogens expressing Tfp include Neisseria gonorrhoeae, Neisseria meningitidis, Vibrio cholerae, Pseudomonas aeruginosa, and enteropathogenic strains of Escherichia coli (8). Tfp contribute to colonization by virtue of promoting binding to epithelial cells, motility-generating capacity, and the ability to aggregate organisms into multicellular infectious units. Tfp are also implicated in horizontal gene transfer by acting as phage receptors and being involved in many instances in competence for natural genetic transformation processes. Full comprehension of the functional relationships underlying these processes can only be achieved in the context of a thorough understanding of the structures and chemistry of Tfp, their subunit proteins, and associated molecules. For N. gonorrhoeae and N. meningitidis Tfp, such concerns are complicated by the extensive antigenic variability of the PilE pilin subunit that arises due to gene conversion-like events between a single expression

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[5]The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1 and Table 1.

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[8]The abbreviations used are: Tfp, type IV pilus; PE, phosphoethanolamine; PC, phosphocholine; DATDH, Gal[1-4]Gal[1-3]2,4-diacetamido-2,4,6-trideoxyhexose; Q-Tof, quadrupole time-of-flight; LPS, lipopolysaccharide; ESI, electrospray ionization; MS, mass spectrometry; MS/MS, tandem MS; MS5, MS/MS/MS or multiple stage MS; HPLC, high performance liquid chromatography; LC, liquid chromatography.
locus and multiple, variable partial gene copies (9). The high degree of PilE antigenic variation likely contributes to both the failure of gonococcal infection to engender protective immunity (10) and the lack of efficacy associated with N. gonorrhoeae Tfp-based vaccines (11).

Post-translational modifications also provide potential sources for PilE structural and functional diversification. The PilE subunit of the N. gonorrhoeae Tfp colonization factor undergoes differential post-translational modifications with phosphoethanolamine (PE) and phosphocholine (PC) at serine 68 (12). This finding is remarkable because these moieties had not previously been observed to be directly O-linked to polypeptide. The presence of PC on pilin was particularly compelling because this moiety is a surface constituent of many microbial pathogens, although in all the other systems it is linked through a carbohydrate moiety. The PC moiety in other bacterial pathogens has been shown to promote epithelial and endothelial cell adherence through binding to the platelet-activating factor (PAF) receptor (13–15) and to act as an immune recognition target for both C-reactive protein (14, 16) and PC-recognizing antibodies (17). In the case of parasitic nematodes, down-modulation of the immune response is attributed to PC-containing glycoproteins (18, 19). In the case of N. gonorrhoeae, the documentation of PC provided a structural basis for the ability of N. gonorrhoeae pilins to react with the TEP-1 monoclonal antibody recognizing a PC epitope (20). A serine-linked phosphoglycerol has also been documented as being localized at residue 93 on N. meningitidis pilin (21). In this work we use the general term phospho-forms to encompass PE and PC pilin modifications.

N. gonorrhoeae and N. meningitidis PilE have also been shown to be glycosylated with N. gonorrhoeae strain N400 subunits bearing a disaccharide composed of a hexose residue linked to a proximal 2,4-diacetamido-2,4,6-trideoxyhexose sugar (HexDATDH) at Ser68 (12) and N. meningitidis strain C311 pilin bearing the trisaccharide Gal(1–3)Gal(1–4)2,4-diacetamido-2,4,6-trideoxyhexose (DATDH) at a serine or threonine between residues 50 and 73 (22). N. gonorrhoeae PilE glycosylation requires at least three gene products highly related to those implicated in the biosynthesis of the proximal bacillosamine component of N-linked glycans in Campylobacter jejuni glycoproteins (12). The mass data for N. gonorrhoeae and N. meningitidis pilin DATDH are consistent with bacillosamine, although its stereochemistry has not been resolved.

An important aspect of PilE phospho-form modifications relates to the enzymatic pathways responsible, and given their novelty, there are no direct precedents for a biosynthetic pathway. In addition, the lic gene family members implicated in PC decoration of LPS and glycoconjugates in other species are absent in both the N. gonorrhoeae or N. meningitidis genomes (23). Taken together with the previous demonstration that phosphoglycerol is a substituent of N. meningitidis pilin, it is noteworthy that all three substituents can be found as the polar head group of eubacterial phospholipids. It is also known that in E. coli, the inner core of LPS is decorated with PE derived from phosphatidylethanolamine in a reaction requiring EptB (24) and that the genomes of most Gram-negative species contain multiple genes whose products are structurally related to EptB. Such gene products directly implicated in PE modification of LPS include Salmonella enterica CptA (25) and PmrC (26) as well as N. meningitidis LptA (27), Lpt3 (28), and Lpt6 (29). Because high frequency frame shifting events within dca (an additional N. meningitidis gene encoding an EptB homologue (30)) was correlated with phase (on-off) variation of the PilE PC epitope, it was renamed pptA as pilin phosphorylcholine transferase A (31). The structural basis for the PC epitope on N. meningitidis pilin remains unknown, although we presume it likely to be the same as described in N. gonorrhoeae pilin. Whatever the case, it remains unclear as to whether the mechanisms for PC and PE modification in N. gonorrhoeae are related to one another.

The structural data for all PilE covalent modifications have been generated by a bottom up approach in which proteolytically derived peptides were examined by electrospray ionization (ESI) mass spectrometry (MS) and tandem MS (MS/MS). Although MS and MS/MS of tryptic peptides are powerful tools for identifying post-translational modifications, they do have limitations. For example, some peptides and post-translational modifications may escape detection due to intrinsic properties relating to ionization efficiency or low stoichiometric abundance, and certain post-translational modifications may exhibit selective instability during digestion and sample preparation (at the level of small peptides). Quantitation using these techniques can also be problematic. As such, the stoichiometries of various modified forms of PilE in Tfp remain unknown, and the full extent of PilE post-translational modifications may have yet to be realized. The latter concern is made more plausible in light of data showing that at least one residue of PilE other than Ser68 is capable of being modified with PC, although the proportion of molecules with the second site modification(s) is low (12).

Top-down MS approaches, in which post-translational modifications can be detected directly from intact proteins, have the potential to alleviate many of the concerns noted above. For example, tandem MS analysis of intact bacterial flagellin proteins has recently been used in the identification of unusual oligosaccharide structures (32). Here, we empirically developed both pilus/pilin preparation schemes and ESI MS methodologies such that intact PilE protein species could have yet to be realized. The latter concern is made more plausible in light of data showing that at least one residue of PilE other than Ser68 is capable of being modified with PC, although the proportion of molecules with the second site modification(s) is low (12).

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Mutants—The bacterial strains used in this study are described in Table 1. E. coli and gonococcal strains were grown as described (33). E. coli HB101 was used for plasmid propagation and cloning experiments. To introduce an alanine substitution at residue 68 of PilE, the Bsu36I-StuI fragment from the plasmid pIga::pilES68A (12) was...
cloned into pPiIE (34) digested with Bsu36I-StuI to give the resulting plasmid pPiIE66A. The S68A substitution creates a new restriction enzyme site (Fnu4HI) that was used to verify the transformation into gonococcal strains by transformation and selection on Gc agar plates containing 10 μg ml⁻¹ chloramphenicol. Introduction of the cat cassette into dca was verified by PCR reactions using genomic DNA from representative transformants and the dca specific primers dca_5' and dca_3'.

Construction of Gonococcal Strains Ectopically Expressing dca—Based on DNA sequence derived from the FA1090 genome project, the complete dca open reading frame plus 422 bp of upstream sequence was amplified by the primers dca_5'SacI (5'-ACTAGAGCTCGATTGGGAAAAAGCATC-CTCCAC-3') and dca_3'SacI (5'-ACTAGAGCTCAGCTG-CTTCCGAATATCG-3') (SacI sites are underlined). The SacI-digested PCR product was cloned into the plasmid p2/16/1 (34) digested with SacI. The resulting derivative p2/16/1_dca was used to reintroduce dca into the iga locus of strains GD2 (dca::kan) and GD3 (pilVfs, dca::kan) by transformation and selection for the linked ermC marker (8 μg ml⁻¹), generating the strains GD4 (dca::kan, iga::dca) and GD5 (pilVfs, dca::kan, iga::dca), respectively. The ectopic localization of dca was confirmed by a PCR reaction using genomic DNA from representative transformants and the primers p2/16/1_5' (5'-GCTAAACAGCCGATTCCAC-3') and p2/16/1_3' (5'-CCTTTTTCCTGAGCGATTCAAG-3'), which are specific for the iga locus (data not shown).

TpP Purification, SDS-PAGE, and Immunoblotting—Pilus purification was carried out as described (37). Procedures for SDS-PAGE and immunoblotting have been described previously (33). PiIE was detected by immunoblotting of whole cell lysates using rabbit polyclonal antibodies and alkaline phosphatase-coupled goat anti-rabbit antibodies (Tago, Inc.). PiIE-specific sera have been described previously (38). PC-decorated proteins were detected by using a 1:1000 dilution of the monoclonal antibody TEP-15 (Sigma) and alkaline phosphatase-conjugated goat anti-mouse IgA (Sigma) (12).

CNBr Cleavage of PiIE—Isolated PiIE proteins were dissolved in 70% formic acid (2 mg/ml) and subjected to CNBr cleavage by adding equal volumes of CNBr solution (50 μg ml⁻¹) and 150 μl endoproteinase V8, sequencing grade, both V8, sequencing grade, both (Promega). Cleavage was carried out as described (36, 37). PiIE was digested with endoproteinase V8 for 16 h at 37 °C. The CNBr-digested PiIE was isolated by ethanol precipitation, washed with ether, and subjected to SDS-PAGE. CNBr-decorated proteins were detected by using a 1:1000 dilution of the monoclonal antibody TEP-15 (Sigma) and alkaline phosphatase-conjugated goat anti-mouse IgA (Sigma) (12). CNBr Cleavage of PiIE—Isolated PiIE proteins were dissolved in 70% formic acid (2 mg/ml) and subjected to CNBr cleavage by adding equal volumes of CNBr solution (50 μg in 100 μl of 70% formic acid). After 24 h of incubation at 37 °C in the dark, CNBr was removed and trapped in NaOH pellets by repeatedly vacuum-drying the samples. CNBr-free samples were dissolved in 5% formic acid and were subject to mass spectrometric analyses or frozen at −80 °C.

In-gel PiIE Digestion—Coomassie-stained protein bands corresponding to PiIE were serially rehydrated and washed with 150 μl of HPLC grade water, 150 μl of acetonitrile/water 1:1 (v/v), and 100% acetonitrile at room temperature. Protein reduction was carried out by the addition of 75 μl of 10 mm dithiothreitol, 0.05 M NH₄HCO₃ (60 min, 56 °C) to the dehydrated gel pieces. Then excess reduction buffer was removed, and thiol groups were alkylated by adding 50 mm iodoacetamide, 0.05 M NH₄HCO₃ (45 min at room temperature in the dark). The gel pieces were washed again with 2 × 150 μl of acetonitrile/water 1:1 (v/v) and 100% acetonitrile at room temperature. Digestion buffer (5–10 μl) containing either 25 ng/μl trypsin (pig, modified, sequencing grade) or 125 ng/μl endoproteinase Glu-C (from Staphylococcus aureus V8, sequencing grade, both from Sigma-Aldrich) in 0.05 M NH₄HCO₃ was added, and samples were kept on ice for 30 min to allow rehydration of the gel pieces. To limit autoproteolysis of trypsin, the remaining buffer

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**TABLE 1**

| Strain   | Parent strain | Relevant genotype | Reference |
|----------|---------------|-------------------|-----------|
| VD300*   | MS11          |                   | Koosney and Falkow (43) |
| N400     | recA6         |                   | Tanjum and Falkow (44) |
| GV1      | N400          | pilV<sup>α</sup>   | Winther-Larsen et al. (45) |
| GV37     | N400          | pilV<sub>α</sub>   | Hegge et al. (12) |
| GD1      | N400          | dca::cat          | Snyder et al. (30) |
| GD2      | N400          | dca::kan          | Snyder et al. (30) |
| GD3      | N400          | dca::kan          | Snyder et al. (30) |
| GE68     | N400          | pilV<sub>α</sub>   | This study |
| GE68V    | GE68          | pilV<sub>α</sub>   | This study |
| GD4      | GD2           | dca::kan, iga::dca| This study |
| GD5      | GD3           | dca::kan, iga::dca| This study |
| GE60     | N400          | pilV<sub>α</sub>   | This study |
| GE63D    | GE63          | pilV<sub>α</sub>   | This study |

*VD300 is an Oppα derivative of MS11.

<sup>*</sup> recA6 is an isopropyl-β-D-galactopyranoside-inducible allele of recA.

<sup>a</sup> pilV<sub>α</sub> is a null allele of pilV with a frame-shift mutation at the G<sub>1</sub> codon (Winther-Larsen et al. (45)).
was removed and replaced with 35–50 μl of 0.05M NH₄HCO₃, and digestions were carried out overnight at 37 °C. Peptides were extracted successively with 5% formic acid and 5% formic acid, acetonitrile (1:1, v/v) and acetonitrile. The combined supernatants were dried by SpeedVac and then re-dissolved in 5% formic acid. Samples were subjected immediately to mass spectrometric analyses or frozen at −80 °C.

Sample Preparation for Intact Protein MS—Isolated PilE proteins were cleaned with a methanol/chloroform precipitation procedure, as described by Wessel and Flügge (39). Briefly, 100 μl of the aqueous PilE solution at 2–3 mg/ml protein was diluted 1:3 (v/v) with methanol and mixed briefly. Both 100 μl of CHCl₃ and 200 μl of water were added consecutively and were followed each time by a mixing step. Phase separation was achieved by centrifugation (4000 g for 8 min), yielding precipitated PilE at the interface. The upper methanol/water phase was removed, and 400 μl methanol was added. After mixing, the pellet was recovered by centrifugation (13,000 g, 8 min). The pellet was dried for 5 min in the inverted tube before dissolving the sample in 50 μl of water, 70% formic acid, acetonitrile 3:1:3 (v/v/v). Samples were subjected immediately to mass spectrometric analyses or frozen at −80 °C.

Infusion MS Analysis of Intact PilE—All data were acquired on a quadrupole time-of-flight mass spectrometer (Q-Tof micro, Micromass, Manchester, UK) equipped with the standard Z-spray ESI source. Sample solutions were infused into the ESI source at a flow rate of 5 μl/min using a syringe pump (Cole-Parmer Instrument Co., Model SP 100i, Vernon Hills, IL). The source block temperature was maintained at 80 °C. Nitrogen was used as both desolvation and nebulizing gas with flow rates of 300 and 20 liters/h, respectively. Mass spectrometric analyses were performed in the electrospray positive mode with the following parameter settings (parameter names as used in the MassLynx NT software, Version 3.5): capillary voltage, 3000 V; sample cone voltage, 25 V; extraction cone voltage, 4.3 V; ion energy, 3 V; collision energy, 10 eV. Mass spectral resolution was typically 4000. The MS Survey was obtained in a mass range from 150 m/z to 1700 m/z. Mass calibration in a mass range of 100–2200 m/z was performed using the ES tune mix solution from Agilent (Palo Alto, CA). The MS spectra were analyzed using the MassLynx software (Version 3.5). For deconvolution, spectra were processed with the MaxEnt1 program of the MassLynx software.

Nanoflow On-line Liquid Chromatographic MS Analysis of Proteolytic and CNBr Peptides—Reverse phase (C18) nano online liquid chromatographic MS/MS analyses of proteolytic or CNBr peptides were performed using a HPLC system consist-

FIGURE 1. Intact mass analyses of N. gonorrhoeae PilE pilin and its CNBr-derived peptide-3. Infusion-positive ESI analysis was done using the Q-Tof mass spectrometer. A, m/z spectrum over a range of 150–1700 m/z showed a charge-state distribution from +11 to +24. Oxonium ions for hexose, hexose-DATDH, and acetylated hexose-DATDH are seen. Note that the site of acetyl linkage to the disaccharide has yet to be defined. B, a deconvolution algorithm was applied to the multiple charged spectrum of A, and a deconvoluted molecular weight spectrum was generated. Multiple forms reflect species differing in glycosylation and PE modification. A complete list of m/z forms and corresponding species of PilE is found in supplemental Table 1. C, a deconvoluted MW spectrum of the CNBr-derived peptide-3 of wild type PilE showing the presence of both unmodified and single-PE-modified forms (minor peaks in the MS spectra toward the higher mass region of m/z 7330 and 7453 are caused by metal-ion adducts (Na⁺, +23 Da; K⁺, +39 Da) or by peptide formylation (+28 Da).
Nano on-line liquid chromatographic MS/MS experiments conducted on the Q-Tof were run under the same settings as described for infusion whole protein MS with the exception that the collision energy in MS/MS experiments were optimized for the corresponding peptides and ranged from 14 to 45 eV. Alternative LC-MS\textsuperscript{e} experiments (n = 2, 3) were conducted on the Bruker Esquire quadrupole ion trap instrument equipped with a manufacturer’s online nanospray interface that was fitted with a distal metal-coated fused-silica PicoTip needle (PicoTip, FS360-20-10-D5-C7; New Objective, Woburn, MA). The ionizer and ion transfer optics parameters were as follows: capillary voltage, $-1500 \text{ V}$; end plate, $-1000 \text{ V}$; capillary exit, 238 V; skimmer, 40 V; octopole I offset, 7 V; octopole II offset, 2 V; octopole radio frequency, 200 V; peak-to-peak, $-5 \text{ V}$ (lens I) and $-60 \text{ V}$ (lens II). The drying gas flow rate was 3 liters/min, the drying gas temperature was 220 $^\circ\text{C}$, and the trap drive was set to 61. Using ESI in positive ion mode, mass spectra were acquired from $m/z = 250$ to 1600. All MS experiments were performed using the ion charge control facility with an accumulation time of 300 ms and with an ion target of 100,000. In MS\textsuperscript{e} experiments (n = 2, 3), precursor ion selection was performed manually, and the ion trap was operated in “smart fragmentation mode,” whereby the fragmentation amplitude of 0.8 V was ramped from 30 to 200% over a period of 40 ms. Data were acquired by the Compass software (Version 1.0) and processed by the BioTools software (Version 1.1).

RESULTS

Intact Mass Analysis of PilE, the N. gonorrhoeae Tfp Pilin Protein—Sample preparation conditions allowing electrospray ionization of intact pilin protein were empirically established. Analysis of purified Tfp from N. gonorrhoeae N400 revealed a broad, heterogeneous envelope of multiply charged ions extending over a range of 500–1700 $m/z$ as well as the presence of oxonium ions for DATDH and HexDATDH at $m/z$ 229.1 and 391.2, respectively (Fig. 1A). A prominent signal was also observed at $m/z$ 433.2, and further analysis has shown that this species is likely HexDATDH modified by acetylation (acHexDATDH, 391.2 + 42).\textsuperscript{5} The reconstructed molecular mass profile of the corresponding spectrum indicated a remarkably broad distribution of modified forms (Fig. 1B). The most abundant species observed in the mass spectrum of intact PilE correspond to the protein modified with a single acHexDATDH.

\textsuperscript{5} F. E. Aas, W. Egge-Jacobsen, H. C. Winther-Larsen, C. Lövold, P. G. Hitchen, A. Dell, and M. Koomey, unpublished data.
moiety and PE at \( m/z \) 17733, consistent with earlier observations (12). Additional modified PilE forms were also observed that could be assigned to pilin protein differentially modified with PE alone and in combination with DATDH or Hex-DATDH moieties of \( m/z \) 17301, 17529, and 17691. Surprisingly, additional signals were observed that are consistent with the presence of an additional PE modification on PilE. The mass of these forms observed at \( m/z \) 17425, 17652, 17814, and 17857 are consistent with the addition of a further PE mass increment. Thus, PilE in purified Tfp from this wild type background is detected as a mixture of single- and double-PE modified forms.

Identification of Ser156 as a Second Site for Covalent Modification of PilE—LC-MS analyses of tryptic digests of PilE failed to reveal any peptides other than 57WPENNT-SAGVASPPTDIK(72 (encompassing Ser156) with a characteristic shift in mass of 123 Da. Therefore, PilE was digested with Glu-C, and the resulting proteolytic fragments were analyzed by LC-MS. Here, two hydrophilic peptides were observed that eluted close to one another with retention times of 35.6 and 35.7 min. Fig. 2 shows a total ion chromatogram of Glu-C digestion fragments as well as two selected ion chromatograms, chosen based on the predicted mass of the Glu-C-derived proteolytic peptide 141IDTKHLPSPTCRDKASDAK(159 either unmodified or modified with PE (both peptides contain also carboxyamidomethyl cysteine instead of cysteine). Both eluting peptides showed four major signals in their mass spectra corresponding to multiply charged molecular ions: 35.6 m, \( m/z \) 1084.0 \( [M+2H]^{2+} \), \( m/z \) 722.7 \( [M+3H]^{3+} \), \( m/z \) 542.2 \( [M+4H]^{4+} \); 35.7 m, \( m/z \) 1022.0 \( [M+2H]^{2+} \), \( m/z \) 681.7 \( [M+3H]^{3+} \), \( m/z \) 511.7 \( [M+4H]^{4+} \), \( m/z \) 409.4 \( [M+5H]^{5+} \). The mass difference between the two sets of ion series derived from the eluted peptides is consistent with PE. The identified candidates were then subjected to further MS/MS and MS3 experiments to confirm the identity of the peptide and the exact site of modification. The modified peptide candidate lost predominantly 141 Da when subjected to low collision energy MS/MS experiments in a Q-Tof mass spectrometer (Fig. 3) or the ion trap (Fig. 4B), suggesting its modification with PE. The mass difference of 141 Da observed between \( m/z \) 2165.9 and 2025.0 corresponds to the loss of PE, concomitantly with 1 molecule of water resulting in the formation of a dehydroalanine residue in the peptide chain. Only small signals within the MS spectra corresponded to the loss of either ethanolamine or PE without additional water. The dominant loss of 141 Da was charge state independent in both instruments. Among all charge states subjected to collision-
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induced dissociation MS/MS analyses, the doubly charged ions of the modified peptide generated spectra with the best quality, showing more peptide backbone fragmentation than other spectra in various charged states (valid for both instruments). Although a complete series of y-ions was not observed, partial backbone fragmentation during Q-Tof analysis allowed unambiguous identification of the peptide as the C-terminal Glu-C cleaved residue of PilE (Fig. 3). In addition, localization of PE to Ser156 was derived by detection of fragment ions y5 and y6 at m/z 543.2 and 601.3, representing a mass difference of 69 Da, corresponding to a dehydroalanine residue formed by the loss of PE from Ser156. The y5 and y6 ions at m/z 543.2 and 601.3 confirm dehydroalanine at position 156, the amino acid produced by the loss of 141 Da (β-elimination at the PE-modified serine).

At different serine or threonine residues within the peptide chain. In summary, these results showed that Ser156 is a second site for PilE covalent modification with PE.

**Influence of an Alanine Substitution at Residue 68 on PilE Phospho-form Modification**—Given the evidence for PE modifications at both Ser68 and Ser156, it was of interest to determine the relative stoichiometries of modifications at these sites. For example, in the case of the single-modified PilE forms seen in the intact protein spectra, it is not clear what fraction of these species was accounted for by those with modification at residue 68 versus those modified at residue 156. It is also unknown if such species might be composed of molecules with PE modification at yet unidentified residues. To begin to address these concerns, PilE protein from wild type Tfp was digested with cyanogen bromide, and the C terminal fragment (termed CNBr-derived peptide-3 in its unmodified and single-PE-modified forms). As another means to assessing the stoichiometries of PE modifications, intact mass analysis of PilE from the strain expressing PilE with an alanine substitution at residue 68 (carrying the pilE<sub>S68A</sub> allele) was performed. The reconstructed molecular mass profile here was devoid of any species repre-
senting PilE bearing two PE modifications, consistent with Ser\(^{68}\) being the predominant site for PE modification (Fig. 5). Furthermore, the profile of CNBr-derived peptide-3 species from this background was nearly identical to that seen for the corresponding wild type preparation save for a slight reduction in the relative level of the single PE-modified form at \(m/z\) 7453. Taken together, these data are consistent with wild type Tfp protein PilV influences phospho-form modification at Ser68 (12). Therefore, phospho-form hypomodification associated with pilV strain GD3 \(pilV::kan\) and GD5 \(pilV::iga\) revealed a spectrum consistent with reduced phospho-form modification at both Ser68 and Ser156 together with a second population bearing only the modification at Ser68. Furthermore, the profile of CNBr-derived peptide-3 species corresponding relative increase in a PE-modified form (\(m/z\) 7330 and 7453) is found in supplemental Table 1. Note that minor peaks in the CNBr-derived peptide-3 MS spectra toward the higher mass region of \(m/z\) 7330 and 7453 are caused by metal-ion adducts (Na\(^+\), +23 Da; K\(^+\), +39 Da) or by peptide formylation (+28 Da).

**FIGURE 6.** Dca (PptA) is required for modification of PilE with both PE and PC. Deconvoluted molecular weight spectra from intact PilE ESI mass spectroscopic analyses and their corresponding deconvoluted MW spectra of the CNBr-derived peptide-3 are shown. Panel A, strain GD2 \(dca::kan\); panel B, strain GD3 \(pilV, dca::kan\); panel C, strain GD4 \(dca::kan, iga::dca\); panel D, strain GDS \(pilV, dca::kan, iga::dca\). A complete list of PE forms and corresponding species of PilE is found in supplemental Table 1. Note that minor peaks in the CNBr-derived peptide-3 MS spectra toward the higher mass region of \(m/z\) 7330 and 7453 are caused by metal-ion adducts (Na\(^+\), +23 Da; K\(^+\), +39 Da) or by peptide formylation (+28 Da).
recognizing the PC epitope (Fig. 6). Intact mass analyses of PilE from two strains bearing different dca (pptA) null alleles were virtually identical to one another, with both devoid of any signals characteristic of phospho-form-modified species (Fig. 6A and supplemental Fig. S1). Signals for PilE derived from a dca (pptA) pilV double mutant were identical to those seen for the dca (pptA) mutants (Fig. 6B). To ensure that the lack of phospho-form modification was due to the absence of Dca (PptA) and not polar effects of the mutations used, PilE from a dca(pptA) mutant strain, which carried a wild type allele at an ectopic site, was examined. Here only modest signals reflecting single PE-modified forms were found (Fig. 6C) but when complementation was examined in a pilV background, these signals were remarkably increased (Fig. 6D). Although no signals characteristic of PC modification were seen, the PC-recognizing monoclonal antibody reacted with PilE in this background (Fig. 7). Together, these results demonstrated unequivocally that Dca (PptA) is required for PilE post-translational modification with both PE and PC and that pilV is genetically downstream of dca (pptA) in influencing phospho-form modification.

**Influence of an Alanine Substitution at Residue 63 on PilE Phospho-form Modification**—Both MS data on tryptic peptides generated from strain N400-derived PilE used here (12) and x-ray crystallographic data of a variant strain PilE (40) demonstrated the presence of a disaccharide linked to Ser63. To assess if glycan occupancy at this site might influence phospho-form modifications, intact mass analyses was carried out on pilin from a strain expressing PilE with an alanine substitution at residue 63 (carrying the pilEs63A allele). Here, the reconstructed molecular mass spectrum indicated a limited distribution of PilE modified forms at m/z 17285 and 17408, which can be accounted for solely by the presence of one or two PE modifications with a minor signal at m/z 17162 reflecting unmodified PilE (Fig. 8A). Interestingly, the results also revealed a shift in which species with two PE modifications predominated that was confirmed by a concurrent increase in the levels of PE-modified CNBr-derived peptide-3 (Fig. 8A, inset). Finally, intact mass analysis of Tfp-derived PilE from a strain expressing the pilEs63A allele in a dca/pptA background revealed a profile corresponding to nonglycosylated PilE devoid of phospho-form modifications (Fig. 8B). Because levels of Tfp were undiminished in this as well as the other backgrounds tested (data not shown), abolition of glycosylation and/or phospho-form modifications do not grossly perturb PilE assembly proficiency.

**DISCUSSION**

PE and PC are increasingly being seen as substituents of surface glycoconjugates in a variety of Gram-negative and Gram-positive pathogens. Although the importance of YhjW/YjdB/ YjIP protein family members in the addition of PE to the core and lipid A of LPS is well established, the enzyme(s) that generates phospho-form-modified *N. gonorrhoeae* Tfp has remained unknown. Here, we show that *N. gonorrhoeae* pptA, nee dca, is necessary for PilE phospho-form modification and propose accordingly that PilE phospho-form modification is mechanistically and evolutionarily related to LPS PE modification systems. Such a model is remarkable given that in one case covalent linkage occurs through a hydroxyl group at serine residues, whereas in the other it occurs at hydroxyl groups on sugars within the core and lipid A domains of LPS. It is also extremely interesting that although both pathogenic and commensal *Neisseria* species have found it advantageous to display PC at their surfaces, the former does it in the context of LPS and utilizes the gene gain/loss
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events occurring during Neisseria evolution and the relative benefits that accrued from them.

Although it is attractive to assume that PptA acts as a PE transferase as proposed for EptB (formerly YhjW), some caution may be warranted. This assumption is based in most instances solely on genetic evidence; that is to say that null mutations at corresponding loci are associated with the absence of PE in target molecules. The exception is eptB, which when overexpressed yielded high levels of membrane-associated PE transferase activity for Kdo2-lipid A (24). Here in this work reintroduction of a pptA gene copy at an ectopic locus in the pptA null mutant restored PE and PC modification, albeit not to levels seen in wild type cells. This result is attributable to reduced level of pptA expression from the ectopic site since it lacks its normal promoter. Nonetheless, this finding formerly rules out polarity on distal gene expression as a contributor to the phenotype seen in N. gonorrhoeae pptA mutants.

Comparisons of PptA and EptB as PE transferases also suggest that common precursors for PE may be involved. In the case of EptB, this is undoubtedly phosphatidylethanolamine, as it is required for transferase activity, and stoichiometric levels of dialcylglycerol are generated during the reaction (24). In pathogenic Neisseria phosphatidylethanolamine is the major membrane phospholipid species, but phosphatidylcholine has only been detected once, and there it was reported to constitute only 3% of the phospholipid profile (41). A more recent study of N. gonorrhoeae phospholipids using fast atom bombardment-MS and gas liquid chromatography-MS technologies did not note the presence of phosphatidylcholine (42). In light of these data, one cannot rule out the possibility that the role of PptA in PilE modification with PC is indirect. In such a scenario PptA would decorate PilE with PE that would subsequently be modified into PC by the action of PE methyltransferase(s). The designation of PptA as a pilin PC transferase is, therefore, not substantiated by current data. Nonetheless, we support the retention of this nomenclature with the modification that PptA corresponds to pilin phospho-form transferase A.

Our findings also demonstrate that PilE undergoes multisite modifications requiring PptA. As documented by MS and reactivity with the PC epitope recognizing monoclonal antibody TEPC-15, both Ser68 and Ser156 are capable of being modified with either PE or PC. It is also important to note that Ser156, in contrast to Ser68, maps in a variable domain of pilin subject to amino acid substitutions. Thus, changes in PilE primary structure can directly impact phospho-form modification by creating and eradicating occupancy sites. Likewise, it was possible to assess the relative degree of occupancy at these sites by PE and PC by quantitation of ion currents of various intact PilE forms standardized against one another. Thus, in a wild type background, PilE in purified Tfp is composed of a mixture of single- and double-PE-modified forms in which modification at Ser68 predominates. Remarkably, the pilin-like protein PilV profoundly influenced modification at both sites. Given the hyper-modification seen in the pilV background and hypomodification seen for the pilVo background, PilV appears to impact on the system by inhibiting PptA-mediated modification. It remains to be determined whether PilV exerts its effects by acting directly on PptA, influencing accessibility of PilE to PptA or through some other controlling mechanism.

Changes in PilE primary structure were also associated with alterations in phospho-form modifications. In addition to the profiles anticipated from the absence of a major occupancy site, substitution of alanine for serine at residue 68 led to reduced occupancy at Ser156, and a similar effect was seen for the Ser68 substitution mutant in a pilVo background. These findings are consistent with a hierarchical model in which occupancy at Ser68 increases the likelihood for modification at Ser156. In the case of the Ser63 substitution mutant that precluded glycosylation, an opposite effect was seen in which levels of PilE with two PE were increased. In the data derived for both the Ser63 and Ser68 substitutions from intact protein analyses, one cannot rule out that the substitutions themselves influence the profiles indirectly by altering ion signal intensities or other aspects of detection efficiencies. Nonetheless, the data for the CNBr-derived peptide-3 (whose primary structures are unchanged in these backgrounds) nicely document altered site occupancy in these backgrounds. It is also noteworthy that the N. gonorrhoeae PilE PC epitope has been reported to be phase-variable (20). In N. meningitidis, this phenomenon correlated with high frequency, frame-shifting events within a monotonous stretch of G residues in pptA, but N. gonorrhoeae pptA lacks this stretch of residues (and, thus, presumably does not undergo high frequency, frame-shifting). Therefore, there must be other factors responsible for this phenomenon. We have observed that in some instances the PC epitope can be detected despite the expression of PilV and, conversely, that phospho-form hyper-modification does not always ensue from pilVo null mutations. In all these cases the altered behavior is associated with expression of a novel PilE variant arising by gene conversion. Like single substitutions within a conserved domain (see above), it appears that changes in the variable domains of PilE arising by combinatorial diversification can also influence phospho-form modification patterns.

Another observation here relates the increased signals corresponding to intact, non-glycosylated PilE forms seen in the pilESer63, pilVo and pptA backgrounds, each of which is associated with decreased or abolished phospho-form modification at Ser68. We consider two explanations for these correlations, one in which phospho-form-site occupancy at Ser68 in some way promotes glycosylation and a second in which Ser68-site occupancy stabilizes the covalent carbohydrate linkage to withstand in source fragmentation. We currently favor the latter scenario because the levels of glyco-free PilE forms seen for the pilESer63, pilVo and pptA pilus samples can be considerably diminished by altering ionization conditions (data not shown). Whatever the basis for these correlations, the data provide the first evidence for interplay between PilE glycosylation and phospho-form modification and further suggests that the proximity of modifications at Ser63 and Ser68 is not entirely fortuitous.

In summary, N. gonorrhoeae PilE structure is more complex and dynamic than originally conceived based solely on combinatorial gene diversification and earlier structural and MS studies. This situation is likely to have significance for how pilin subunits are assembled and organized into helical filaments, particularly if one assumes that phospho-form sites are exposed on the pilus surface. It follows then that PC and PE and reagents...
recognizing them may serve as valuable probes to assess PilE topology within Tfp. Other important facets remaining to be addressed concern the catalytic mechanisms of PptA and the identification of the precursors involved. Here, insights into the properties and structure of PptA will be pivotal to characterizing the pathway and its elements. Finally, the ability to produce structurally defined and altered PilE molecules should help elucidate the potential contributions of post-translational modifications to Tfp-associated functions and phenotypes.

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