Variable processing of the IgA protease autotransporter at the cell surface of Neisseria meningitidis

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As with all classical monomeric autotransporters, IgA protease of Neisseria meningitidis is a modular protein consisting of an N-terminal signal sequence, a passenger domain and a C-terminal translocator domain (TD) that assists in the secretion of the passenger domain across the outer membrane. The passenger of IgA protease consists of three separate domains: the protease domain, the γ-peptide and the α-peptide that contains nuclear localization signals (NLSs). The protease domain is released into the extracellular milieu either via autocatalytic processing or via cleavage by another autotransporter, NalP, expression of which is phase-variable. NalP-mediated cleavage results in the release of a passenger that includes the α- and γ-peptides. Here, we studied the fate of the α-peptide when NalP was not expressed and observed strain-dependent differences. In meningococcal strains where the α-peptide contained a single NLS, the α-peptide remained covalently attached to the TD and was detected at the cell surface. In other strains, the α-peptide contained four NLSs and was separated from the TD by an IgA protease autoproteolytic cleavage site. In many of those cases, the α-peptide was found non-covalently associated with the cells as a separate polypeptide. The cell surface association of the α-peptides may be relevant physiologically. We report a novel function for the α-peptide, i.e. the binding of heparin – an immune-modulatory molecule that in the host is found in the extracellular matrix and connected to cell surfaces.

INTRODUCTION

Proteins that are secreted by Gram-negative bacteria have to cross the bacterial cell envelope, which consists of the inner membrane, the periplasm containing the peptidoglycan layer and the outer membrane. The family of classical monomeric autotransporters constitutes a widespread secretion system among the Gram-negatives (Celik et al., 2012). These autotransporters are modular proteins that consist of an N-terminal signal sequence for transport across the inner membrane via the Sec system, a C-terminal translocator domain (TD) and, in between, the secreted passenger domain (Grijpstra et al., 2013; van Ulsen et al., 2014). The TD inserts into the outer membrane, where it forms a 12-stranded β-barrel and facilitates the transport of the passenger across that membrane (Oomen et al., 2004; Roussel-Jaze´de´ et al., 2011; Saurı´ et al., 2011). Secretion further requires the Bam complex, which constitutes the machinery that inserts outer membrane proteins into the outer membrane (Jain & Goldberg, 2007; Voulhoux et al., 2003).

The overall three-domain organization is a general feature of autotransporters, but these domains can be further organized into separate subdomains (Fig. 1a). For example, in the canonical autotransporter IgA protease of Neisseria gonorrhoeae, encoded by the iga gene, the secreted passenger domain is further processed into a protease domain (~106 kDa), a γ-peptide (~3.1 kDa) and an α-peptide (~45 kDa) (Pohlner et al., 1987). Similarly, our laboratory has shown that the passenger domains of the autotransporters App and AusI of Neisseria meningitidis consist of

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Abbreviations: NLS, nuclear localization signal; TD, translocator domain.
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at least two subdomains (van Ulsen et al., 2003, 2006). The TDs may also consist of several subdomains, e.g. the TDs of IgA protease of *N. gonorrhoeae* and AIDA-I of *Escherichia coli* consist of a surface-exposed linker peptide, which is sensitive to externally added proteases, and a membrane-embedded β-core (Klauser et al., 1993; Konieczny et al., 2001). This β-core is protected against proteases and compares to the crystallized TDs of other autotransporters (Roussel-Jazedé et al., 2011).

In *N. meningitidis*, two different and mutually competing proteolytic events were found to be involved in the release of the passenger of IgA protease, resulting in the release of two different variants of the protein (van Ulsen et al., 2003). Autoproteolytic cleavage results in the release of the protease domain as a separate polypeptide. Alternatively, proteolytic processing can be mediated by another autotransporter, NalP, whose expression is prone to phase variation (Roussel-Jazedé et al., 2013; van Ulsen et al., 2003). NalP-released IgA protease passengers include the protease domain and the α-peptide (Fig. 1a).

In *N. gonorrhoeae*, which does not encode a functional NalP (van Ulsen et al., 2001; van Ulsen & Tommassen, 2006), the α-peptide of IgA protease was detected as a separate polypeptide in the extracellular medium (Pohlner et al., 1987). In *N. meningitidis*, the involvement of NalP may indicate different processing and the fate of

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**Fig. 1.** Schematic overview of the various domains and subdomains of neisserial IgA proteases (not drawn to scale). (a) Full-length IgA protease with the possible positions of autocatalytic processing sites and their sequences and the approximate position of the NalP cleavage site indicated. Arrowheads indicate the potential positions of NLSs, whereby the open arrowhead shows the position of the NLS in α-peptides with only a single NLS present. (b) Schematic representation of the differences between α-peptides and linker regions of different *N. meningitidis* and *N. gonorrhoeae* strains. The arrowheads and the colours of the α-peptide and linker region match those in (a). The lighter boxes indicate the positions of repeats sequences: a nonapeptide repeat ([RKAAELLAK] and variants thereof; Jose et al., 2000) in the α-peptide, one or two copies of the peptide [VSESVDTSDKPQDNTELHEKYEN] in the linker and one to four copies of a tetra-peptide repeat ([QAAA] or [QAVA]) at the C terminus.
the IgA protease, when not released by NalP, is unknown. Also, the organization and appearance of the TD after the release of the passenger variants is unknown. However, the TD does show pore activity in vitro (Roussel-Jazédé et al., 2011) and its accumulation in the outer membrane, therefore, might affect the membrane’s integrity. We investigated the fate of the IgA peptide and the TD of meningococcal IgA proteases in the presence and absence of NalP. The studies revealed novel cell-associated fragments of IgA protease, but also showed strain-dependent differences. This differential processing may have functional implications, as the IgA peptide has been implicated in biofilm formation (Arenas et al., 2013), contains nuclear localization signals (NLLs) that function in vitro (Pohler et al., 1995) and binds heparin, as shown in the present study.

METHODS

Bacterial strains and growth conditions. N. meningitidis strains H44/76, its unencapsulated derivative HB-1, 2996 and B166B, and the iga::kan and nalP::kan mutant derivatives thereof, have been described previously (van Ulsen et al., 2003; Bos & Tommassen, 2005; Arenas et al., 2013). Other strains of N. meningitidis and Neisseria gonorrhoeae used are listed in Table S1 (available in the online Supplementary Material) and are from our laboratory collection (van Ulsen et al., 2006). To create a nalP::cam iga::kan double mutant of strain B166B, nalP was first disrupted by allelic exchange through transformation with plasmid pONalP-cat (Arenas et al., 2013), which carried a nalP allele in which an internal 2112 bp fragment was replaced by a chloramphenicol-resistance gene. Subsequently, the iga gene was knocked out by allelic exchange using the iga::kan plasmid described previously (Vidarsson et al., 2005). The strains were grown on GC agar (Oxoid) supplemented with Vitox (Oxoid) at 37 °C in candle jars and liquid cultures were grown in tryptic soy broth (TSB; Gibco-BRL) at 37 °C with mild shaking. Where indicated, N. meningitidis strains H44/76 and B166B were grown in RPMI 1806 medium (Gibco-BRL) supplemented or not with 5% FCS (PAA Laboratories). To obtain heat-inactivated FCS, samples were incubated for 30 min at 56 °C. E. coli strains BL21(DE3) (Invitrogen) and DH5α were grown in lysogeny broth. Antibiotic concentrations added for plasmid maintenance were 100 μg ampicillin ml⁻¹ and 25 μg chloramphenicol ml⁻¹ for E. coli, and 10 μg chloramphenicol ml⁻¹ for N. meningitidis. When appropriate, IPTG was added at a final concentration of 1 mM to induce gene expression.

Sequencing and sequence comparison. DNA fragments encoding the IgA peptide and the linker peptide of the IgA proteases of various N. meningitidis strains were amplified by PCR from genomic DNA using primers IgaA1(5’-CCCGATGGTCAATCCTTATGCAGGCA-3’) and IgaA4(5’-GCCAAATGAGCCGTTTTGGA-3’) based upon iga of N. meningitidis strain MC58. Genomic DNA was prepared from bacteria that were resuspended in water to OD550 = 2.0. The suspension was boiled for 5 min and then centrifuged at full speed in a microfuge. The supernatant was used as template DNA for the PCR, which was performed as described (van Ulsen et al., 2001). Resulting PCR fragments were cloned into pCRII-TOPO (Invitrogen) and sequenced (GenBank accession numbers GU190730-GU190733 and KJ653448-KJ653452). Sequence alignments were performed with the deduced amino acid sequences and using the CLUSTAL Omega program at https://www.ebi.ac.uk/Tools/msa/clustalo/ (Sievers et al., 2011).

Plasmid construction. The iga gene of N. meningitidis strain H44/76 was amplified by PCR from genomic DNA using primers based upon iga of N. meningitidis strain MC58 and cloned in pCRII-TOPO, yielding pCRT_iga_H44/76. DNA fragments encoding the IgA peptide(aa 1005-1182) and the TD (β-core plus linker peptide; aa 1183-1561) were obtained by PCR using the primer couples IgaAstart (5’-CCATATGAGGGCCAGGCAAATCGGCA-3’) and IgaEnd (5’-GGATCTGGGCGGTTGCCAGATGAT-3’) and IgaTD (5’-GCTATGAGGGCCAGGCTGTCAG-3’) and ligated into pCRT_iga_H44/76 as template DNA. PCR fragments were cloned into pCRII-TOPO. The fragments encoding the IgA peptide and the TD were excised from the pCRII-TOPO constructs with Ndel and BgII, and ligated into Ndel/BamHI-digested pET11a, yielding pET_Iga-APHH44 and pET_Iga-TDH44, respectively.

Collection of cells and supernatants. Cells were harvested by centrifugation (4500 g, 5 min) and resuspended in PBS (pH 7.6) to OD550 10. The culture supernatants were centrifuged again (16000 g, 5 min) to remove residual cells. Then, the protein content was precipitated by adding ice-cold TCA to a final concentration of 5% and incubation for at least 30 min at 4 °C. Samples were centrifuged (16000 g, 20 min), and the pellets were washed with 90% acetone and dissolved in PBS. Relative to the OD550 of the original cultures, the precipitated proteins from the culture supernatant fractions were 10-fold more concentrated than the cell lysates. The protein preparations were mixed with an equal volume of twofold concentrated sample buffer and boiled for 10 min.

Electrophoresis and immunoblotting. Protein samples were run on 10% (w/v) SDS-PAGE gels and blotted onto a 0.45 μm Protran membrane (Schleicher & Schuell). Unspecific binding of antibodies to the filters was prevented by overnight incubation in blocking buffer [PBS containing 0.5% non-fat dried milk (Nutricia) and 0.1% Tween 20 (Merck)]. The sera were diluted 1: 5000 or 1: 20,000 in the same buffer and applied for 1 h to the blots. After washing the blots four times for 5 min with blocking buffer, the blots were incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (Biosource) diluted 1: 10 000 in blocking buffer for 1 h. Binding of antibodies was visualized by chemiluminescence using an ECL kit (Amersham).

Antisera. The antisera against the TD of NalP (Oomen et al., 2004) and against IgA protease of strain HFI3 (Vidarsson et al., 2005) have been described previously. To raise antisera against the IgA peptide and TD of IgA protease of strain H44/74, recombinant proteins were purified from IPTG-induced E. coli BL21(DE3) carrying plasmids pET_Iga-APHH44 and pET_Iga-TDH44 as described previously (van Ulsen et al., 2003), and sent to Eurogentec to raise the polyclonal rabbit antisera.

Antibodies specifically recognizing the IgA peptide of IgA protease were further purified from the anti-IgA protease IgA-antiserum by affinity purification. Recombinant IgA-protease was run on a 10% SDS-PAGE gel and electroblotted. The IgA peptide band was localized on the blot by staining in 0.1% Ponceau 5/5% acetic acid and cut out. The blot slice was pre-incubated in blocking buffer and then incubated for 1 h with antiserum, diluted 1: 100 in blocking buffer. After extensive washing, the specifically bound antibodies were eluted by incubating the slice with 0.2 M glycine (pH 3.0) for 5 min. The elution buffer was neutralized by adding 50 μl 1 M Tris/HCl (pH 8.0) and then diluted 10-fold in blocking buffer before use.

Immunofluorescence microscopy. Exponential-phase cultures were centrifuged at 4500 g for 5 min and the cells were prepared for immunofluorescence microscopy as described previously (van Ulsen et al., 2006).

Protease accessibility assays. Bacterial cells were harvested by centrifugation (7000 g, 10 min). Pellets were washed with PBS.
(pH 7.6) and resuspended in 10 mM Tris/HCl (pH 7.6) and 10 mM MgCl₂, to OD₆₀₀ 1.0, and incubated on ice for 10 min. Aliquots of 500 μl of the cell suspensions were incubated with 20 μg trypsin ml⁻¹ or 20 μg proteinase K ml⁻¹ (Roche) for 30 min on ice. Protease activity was stopped by adding 5 μg PMSF ml⁻¹ (Sigma) and further incubation for 30 min on ice. Cells were then harvested by centrifugation and analysed by immunoblotting.

**Cell envelope preparation.** Cells were harvested by centrifugation (7000 g, 10 min) and the pellet was frozen at −20 °C for at least overnight. After thawing, the pellet was resuspended in 50 mM Tris/HCl (pH 8.0) and 2 mM EDTA, and then disrupted by sonication for 2 × 5 min in a Branson sonifier at full power whilst on ice with 5 min between the steps. Lysates were cleared from unbroken cells by centrifugation (1400 g, 10 min). The membrane fraction (mainly outer membranes) was pelleted by centrifugation (8 min, 100 000 g) and resuspended in 5 mM Tris/HCl (pH 7.6).

**Heparin affinity chromatography.** The binding of the α-peptide of IgA protease to heparin was analysed using a 1 ml HiTrap heparin HP column (GE Healthcare). Aliquots of 50 ml culture supernatants of HB-1 and HB-1::kan were dialysed twice against 1 l 10 mM sodium phosphate (pH 7.0) at 4 °C for 2 days. Then, 100 μg DNase I (Sigma) ml⁻¹ was added, and the sample was incubated for 3 h at 37 °C and then centrifuged at 13 000 g for 10 min at 4 °C. The resulting supernatant was loaded onto a pre-washed heparin column and the columns were washed with 40 vols phosphate buffer (pH 7.2). Finally, the heparin-bound protein was eluted with phosphate buffer containing 1.0 M NaCl. The protein content of the culture supernatant and of fractions from the heparin column were concentrated by TCA precipitation and analysed by immunoblotting.

**RESULTS**

**Fate of the α-peptide of IgA protease in a nalP mutant of strain HB-1**

Approximately equal amounts of two forms of IgA protease are released into the extracellular medium of strain HB-1 – an unencapsulated derivative of H44/76. The smaller form of ~110 kDa, representing the IgA protease passenger without the α-peptide attached, is generated by autocatalytic activity, whilst the larger variant with an apparent molecular mass of ~160 kDa in SDS-PAGE gels was generated by NalP activity and represents the protease domain with attached α-peptide (van Ulsen et al., 2003). The calculated molecular mass of the larger variant is only ~136 kDa, but the aberrant electrophoretic mobility is likely caused by the unusually high number of charged residues in the α-peptide rendering it very basic (Pohlner et al., 1995). To determine the fate of the α-peptide after autocatalytic processing, we analysed whole-cell lysates and culture supernatants of strain HB-1 and its nalP::kan and iga::kan mutant derivatives. Immunoblots incubated with an antiserum directed against the TD revealed a band of ~34 kDa in the whole-cell lysate of HB-1 (Fig. 2a). This polypeptide was much smaller than the TD of IgA protease of the gonococcal strain MS11, which has a calculated mass of 45 kDa (Pohlner et al., 1987), but corresponded in size to the β-core of that TD (Klauser et al., 1993). In contrast, in the lysate of the nalP mutant, a ~72 kDa band was detected with the same antiserum as well as with an antiserum directed against the α-peptide (Fig. 2a). This band therefore most likely represented a polypeptide consisting of the α-peptide fused via the linker peptide to the β-core, which together has a calculated molecular mass of 62 kDa. The faint ~170 kDa band detected with the α-peptide antiserum (Fig. 2a, right panel) likely was full-length unprocessed IgA protease present in the cell lysates. When we complemented the nalP mutant with plasmid pEN300, carrying nalP under control of a lac promoter (van Ulsen et al., 2003), the separate β-core fragment was detected in cell lysates after induction of NalP synthesis with IPTG, whereas the non-induced cells showed the ~72 kDa TD with the α-peptide attached (Fig. 2b).

Next, two other N. meningitidis strains, i.e. 2996 and B16B6, and their nalP::kan mutant derivatives were analysed. As in strain HB-1, a band of ~33–34 kDa that reacted only with the antiserum against the TD was detected in whole-cell lysates of the WT strains, whereas a ~73–75 kDa band was detected in the cells of the nalP-mutant derivatives that reacted with both antisera directed against the TD and against the α-peptide (Fig. 2c). In the B16B6 nalP::kan mutant, an additional ~37 kDa band was detected with the antiserum directed against the TD (Fig. 2c); this band might have resulted from processing by another protease present at the cell surface. Unexpectedly, a faint band of ~75 kDa in the lysate of WT 2996 reacted with the anti-α-peptide serum, suggesting that NalP might be less active in this strain or switched off by phase variation in a portion of the cells. The expression of nalP in strain 2996, indeed, appeared relatively low on immunoblot (Fig. S1a), but analysis by immunofluorescence microscopy indicated that this was not due to phase variation in a part of the population, as most cells appeared to express the protein (Fig. S1b). Taken together, these results indicated that the α-peptide of IgA protease remained fused to the TD when NalP was not expressed.

In addition to IgA protease, NalP also processes the autotransporters App and Ausl (van Ulsen et al., 2003, 2006). The passenger of App also includes an α-peptide. To analyse the fate of the α-peptide of App in the absence of NalP, similar immunoblotting experiments were performed on whole-cell lysates of HB-1 and its nalP::kan and app::kan mutant derivatives as described above for IgA protease. The results showed the accumulation of a ~60 kDa band that reacted with both antisera directed against the TD and against the α-peptide of App (Fig. S2), indicating that the α-peptide of App also remained attached to the TD in absence of NalP.

**Variations in autocatalytic processing of IgA protease between meningococcal strains**

The results for the N. meningitidis strains described above deviated from those described for the IgA protease of N. gonorrhoeae MS11, whose TD after passenger release was reported to be ~45 kDa and to consist of two subdomains:
the linker peptide of ~14 kDa and the β-core of ~34 kDa (Klauser et al., 1993; Pohlner et al., 1987). To verify those results, we analysed the membrane-associated IgA protease domains of two gonococcal strains, FA1090 and MS11. Blots containing cell envelopes of these strains were incubated with antiserum directed against the TD and revealed bands of ~45 and ~50 kDa, respectively (Fig. S3), in agreement with the calculated molecular masses of 40 and 45 kDa, respectively. These bands did not react with the anti-α-peptide antiserum (results not shown).

The differences in processing of IgA protease between \( N. \) meningitidis and \( N. \) gonorrhoeae can be explained only in part by the absence of a functional \( \text{NalP} \) in \( N. \) gonorrhoeae (van Ulsen et al., 2001; van Ulsen & Tommassen, 2006). Whilst this absence may explain why the linker peptide is not released from the β-core in the IgA protease of \( N. \) meningitidis, it fails to explain the observation that the α-peptide remained fused to the TD in \( \text{nalP} \) mutants of \( N. \) meningitidis. IgA protease of \( N. \) gonorrhoeae was reported to contain three autocatalytic processing sites, i.e. between the protease domain and the γ-peptide, between the γ-peptide and the α-peptide, and between the α-peptide and the linker peptide (Fig. 1). Inspection of the sequence of IgA protease from strain H44/76 (Budroni et al., 2011; Piet et al., 2011) revealed that the autocatalytic cleavage site between the α-peptide and the linker peptide was missing (Fig. S4). This explained why the α-peptide remains covalently associated with the TD when \( \text{nalP} \) was not expressed.

Analysis of full-length IgA protease sequences in the GenBank database \((n=51)\) revealed a very high sequence diversity that concentrated in the α-peptide and linker regions of the proteins (see Fig. S4 for examples and Table S2 for a list of sequences used). This variability included differences in the numbers of NLSs, and repeats upstream of and in between the NLSs, as well as in the linker. Based on this variability, four different subtypes of α-peptides could be distinguished (see Fig. 1b for a schematic representation), with limited variation within each subtype. The variability also included the presence or not of an autocatalytic cleavage site between the α-peptide and the linker peptide. This cleavage site was present in the IgA proteases of all \( N. \) gonorrhoeae strains \((n=13)\), as well as of many \( N. \) meningitidis strains analysed \((n=25/38)\), including those from the sequenced genomes of MC58 and Z2491 (Fig. S4). However, this site was not present in 13 of 38 meningococcal isolates, including H44/76, B16B6 and FAM18, which were therefore all expected to have in their membranes a TD that included both the linker and α-peptide when \( \text{nalP} \) was in phase “off”.

The sequence analysis suggested that many meningococcal strains, similar to \( N. \) gonorrhoeae, should contain a TD consisting of the β-core and linker, but lacking the α-peptide when NaP is not expressed. To confirm this possibility, we analysed in total 20 \( N. \) meningitidis isolates of which we had previously determined the \( \text{nalP} \) expression status (phase “on” or “off”) by sequencing of the polycytosine stretch where slipped-strand mispairing was expected to occur (van Ulsen et al., 2006; Table S1), and by immunoblotting and immunofluorescence microscopy (see Fig. S1 for examples). Immunoblots containing whole-cell lysates of these strains were then probed with antiserum against the TD of IgA protease. In 12 out of
the 13 NaLP⁺ strains examined, a ~34 kDa band corresponding to the β-core was detected (see Fig. 3a for examples), consistent with the expected release of the linker and χ-peptide. The exception was strain M992, which did express NaLP (Fig. S1) but, nevertheless, showed a predominant ~75 kDa band corresponding to the TD associated with the χ-peptide, as well as bands of ~33–35 kDa that appeared to represent the expected β-core. Apparently, the strain encoded an IgA protease variant that was less-well recognized by NaLP.

Three out of the seven meningococcal strains that did not express nalP revealed a major band of ~75 kDa reacting with the anti-TD antiserum, as in the nalP mutant of strain HB-1 (i.e. strains 35E, FAM18 and M990; see Fig. 3a). Strain FAM18 additionally showed a prominent 37 kDa band (Fig. 3a), as the nalP mutant of strain B16B6 did (Fig. 2c); these two strains are of the same clonal complex, i.e. cc11. Interestingly, four nalP phase “off” strains, i.e. S3446, 881710, 13077 (Fig. 3a) and 881607 (result not shown), showed a predominant 45–50 kDa band, similar to N. gonorrhoeae strains MS11 and FA1090, suggesting the presence of an additional autocatalytic processing site between the χ-peptide and linker of the IgA proteases of these strains. The sequence of the corresponding DNA segments confirmed the presence of this additional autocatalytic cleavage site downstream of the χ-peptide (Fig. S5). We also analysed the corresponding segment of strains 2996 and B16B6, which showed a ~75 kDa band similar to HB-1 (Fig. 2c), and of strain M992, which showed this band even when it expressed NaLP (Fig. 3a). As expected, these sequences lacked the additional autocatalytic cleavage site (Fig. S5).

Blots were also probed with the antiserum against the χ-peptide (Fig. 3b). This antiserum recognized ~72–75 kDa bands in the strains that were positive for a similarly sized band using the antiserum directed against the TD, which confirmed that this band corresponded to the TD with associated χ-peptide. In addition, the antiserum recognized a band of ~49 kDa in the lysates of nalP phase “off” strains that possessed an IgA protease with a confirmed additional autoproteolytic cleavage site between the χ-peptide and the linker peptide (strains S3446, 881710 and 13077 in Fig. 3b; see also Fig. S5). This band, therefore, must represent the cleaved χ-peptide that is of similar size in all these strains (calculated at 44 kDa) and contains four NLSs (Fig. S5). Apparently, these χ-peptides were not released into the medium after they are autotactically cleaved from the TD, but remained non-covalently associated to the cell surface. The cleaved χ-peptide was also detected in the cell lysate of the NaLP-expressing strains MC58 and 2208, but, for unknown reasons, not in those of strains 892557 and M981 (Fig. 3b). In the IgA proteases that did not contain an autoproteolytic cleavage site between the χ-peptide and the linker peptide, such as the IgA proteases of strains HB-1, B16B6 and 2996, a fragment consisting of the χ-peptide and the linker was expected to be the result of a combined autocleavage and NaLP-mediated cleavage. Such a peptide was not detected with the antiserum directed against the χ-peptide in the cell lysates of these strains (Fig. 2a, c). However, examination of the culture supernatant of strain HB-1 revealed the presence of this peptide, which migrated
at ~40–42 kDa (Fig. 3c), which was higher than the calculated ~28 kDa, as observed for other \(x\)-peptide-containing bands. Interestingly, the \(x\)-peptides of the IgA proteases in these strains contained only a single NLS (Fig. S5).

Taken together, our results indicate clearly that in *N. meningitidis* the membrane-associated IgA protease fragment that remains after the release of the passenger can exist in three different forms: (i) the ~34 kDa \(\beta\)-core resulting from NalP-mediated processing, (ii) the 45–50 kDa TD consisting of the \(\beta\)-core with the linker attached or (iii) the ~75 kDa form consisting of the TD with linker, \(\gamma\)- and \(x\)-peptides attached. The latter two forms resulted from autoproteolytic cleavage. In addition, in several strains the autoproteolytically cleaved loose \(x\)-peptide remained non-covalently cell-associated.

**Membrane-associated IgA protease fragments are stable and extend from the cell surface**

The detection of the TD of IgA protease in its various forms on immunoblots suggested that these fragments were not rapidly degraded and, therefore, could be functionally relevant. To verify their stability, heparin columns showed that their TDs were also stable within the 4 h time period from the eluate of the heparin column, most likely because the \(x\)-peptide was released from the protease domain by autoproteolysis during the time-consuming dialysis steps prior to application on the heparin column. Indeed, we observed that the \(x\)-peptide-containing version of the IgA protease passenger was further processed into its separate subdomains during prolonged incubations of supernatant fractions (Fig. S7).

**TD-associated domains are subject to cleavage by serum components**

In the course of experiments in which we investigated the interaction of *N. meningitidis* with cultured eukaryotic cell lines, we noticed that the culture medium could affect the membrane-associated domains of IgA protease. In particular, we detected the presence of a ~43 kDa band in whole-cell lysates of B16B6 *nalP::kan* and HB-1 *nalP::kan* instead of the expected band of ~75 kDa when these strains were grown in RPMI medium supplemented with 5 % FCS, i.e. the medium used routinely in cell culture experiments (Fig. 5b). This 43 kDa band was not a cross-reacting band derived from the medium, as it was not detected in a B16B6 *nalP::cam iga::kan* double mutant (Fig. 5b). The presence of FCS in the culture medium appeared responsible for the generation of the 43 kDa band, as the expected 75 kDa band representing the \(x\)-peptide fused to the TD was observed after growth in RPMI without FCS. Furthermore, samples of cells grown in RPMI supplemented with FCS that was heat-inactivated for 30 min at 56 °C did show the 75 kDa band along with the 43 kDa band (Fig. 5b) and prolonged incubation of FCS at 56 °C to >1 h restored the 75 kDa band as the predominant band, whilst the 43 kDa band was barely detectable any more (result not shown). Similar results were obtained when human serum instead of FCS was used (results not shown). Thus, serum appeared to include a heat-labile proteolytic enzyme that cleaved the cell-associated TD plus \(x\)-peptide fragment of IgA protease within the \(x\)-peptide and/or the linker peptide.

**DISCUSSION**

Expression in *N. meningitidis* strain H44/76 of the phase-variable autotransporter NalP results in the release of IgA
protease passengers extended with the $\alpha$- and $\gamma$-peptides (van Ulsen et al., 2003), and with the linker peptide (this study). We also show here that a 34 kDa fragment remains in the cell envelope. This fragment corresponds to the $\beta$-core (Klauser et al., 1993) consisting of a 12-stranded $\beta$-barrel and an $\alpha$-helix that plugs its channel (Roussel-Jazédé et al., 2011). In the absence of NalP, a shorter variant of the IgA protease passenger is released via autocatalytic cleavage. Our results further show that the sizes and composition of the secreted and membrane-bound domains of IgA protease vary depending on the N. meningitidis strain analysed. The secreted proteins may consist of the extended passenger, or the passenger, $\gamma$-peptide and $\alpha$-peptide, with or without linker peptide attached, as separate proteins. The membrane-bound protein may consist of either a TD as found in N. gonorrhoeae MS11, i.e. the $\beta$-core extended with the linker peptide, or as the separate $\beta$-core, or as the $\beta$-core extended with the linker and the $\alpha$-peptide. Which variant exists is determined by the presence or absence of autocatalytic processing sites, as well as the expression status of the phase-variable nalP gene. The secreted and cell-associated domains of the autotransporter App showed a similar dependence on nalP expression (van Ulsen et al., 2003) (Fig. S2). Furthermore, the different autotransporter domains that remain in the membrane appear very stable (Figs 4 and S6) and, therefore, mechanisms must exist to prevent the membrane from being filled up with these protein fragments. Possibly, N. meningitidis maintains a fine balance between autotransporter production and the dilution of accumulated TDs by cell division. However, the organism is also well known for its abundant release of outer membrane vesicles. Thus, an alternative mechanism could be that these also release the redundant outer-membrane-associated autotransporter fragments. Such a mechanism is supported by a proteomics analysis that indicated that autotransporter proteins were enriched in outer membrane vesicles over isolated membrane fractions (Lappann et al., 2013).

The NalP-mediated release of the $\alpha$-peptide from the cell surface could be a protective measure (Roussel-Jazédé et al., 2010). The $\alpha$-peptide of IgA protease can elicit responses of human T-cells and thus activate the immune system (Jose et al., 2000). Consistently, the $\alpha$-peptide and linker peptide are the most variable parts of IgA protease (Figs 1b, S4 and S5) – a feature often associated with immunodominance. The NalP-mediated release of these fragments from the cell surface could help meningococci to escape the immune response. In the absence of NalP, removal of the $\alpha$-peptide occurs autocatalytically in many strains of N. meningitidis and N. gonorrhoeae, due to the presence of a processing site between the $\alpha$-peptide and linker. Arguing against an immunoprotective function of the release, either autocatalytically or via NalP, is perhaps our observation that the released $\alpha$-peptide remained non-covalently associated with the cells in some meningococci, as found in N. meningitidis MS11, i.e. the $\alpha$-core.
indicating that complete release from the cell surface may not be that important.

Fig. 5. Interaction of the IgA protease z-peptide with host components. (a) Concentrated culture supernatants of strain HB-1 and its iga::kan mutant derivative were dialysed and loaded onto HiTrap heparin HP columns to detect binding. Various fractions of the isolation procedure were analysed by immunoblotting with antisera directed against the z-peptide (zP). First and second wash represent fractions obtained after washing the column with 5 and 40 vols of buffer, respectively. The positions of full-length IgA protease and the z-peptide are indicated. (b) FCS components cleave the cell-associated z-peptide from the TD. Immunoblots of whole-cell lysates of N. meningitidis strains B16B6 (left panel) and HB-1 (right panel) and their "nalP::kan and "nalP::cam iga::kan mutant derivatives were grown in RPMI, RPMI supplemented with 5% FCS (+FCS) or RPMI supplemented with FCS that had been heat-inactivated for 30 min at 56 °C (+HI FCS). Whole-cell lysates were analysed with antisera directed against the TD of IgA protease.

If the z-peptides are highly immunogenic, their ubiquitous presence in the IgA proteases from N. meningitidis and N. gonorrhoeae implies an important role in bacterial physiology or virulence. The z-peptide contains NLSs and the reporter proteins fused to the z-peptide of IgA protease from N. gonorrhoeae have been shown to target the nucleus of transfected cell lines (Pohlner et al., 1995), although functional implications of targeting IgA protease itself to the nucleus remain to be demonstrated. Recently, we showed that the z-peptide of meningococcal IgA protease is involved in biofilm formation, presumably by binding extracellular DNA (Arenas et al., 2013). NLSs are characterized by their high number of positively charged residues that could explain their affinity for DNA or other negatively charged surfaces. In this study, we showed that the z-peptide is able to bind heparin, which is negatively charged as well. Heparin is a known ligand for bacterial adhesins (Duensing et al., 1999; Menozzi et al., 2002). It also interacts with proteins that regulate the complement system, including factor H, C4b-binding protein and C1 inhibitor (Yu et al., 2005), and attaching these factors via heparin to the bacterial cell surface may enhance serum resistance (Duensing et al., 1999; Menozzi et al., 2002). N. meningitidis contains multiple heparin-binding proteins, including the Neisseria heparin-binding antigen NhBA, whose recruitment of heparin via an arginine-rich protein segment that resembles the NLSs in the z-peptides of IgA proteases has been shown to result in increased serum resistance (Serruto et al., 2010). Furthermore, heparin bound via the z-peptide to the bacterial surface might bind vitronectin, thus allowing for uptake of the bacteria into host cells expressing vitronectin receptors (Duensing et al., 1999). In conclusion, the z-peptide of IgA protease appears to have various functions dependent on whether it is released in association with the IgA protease into the milieu or whether it remains associated with the bacterial cell surface. Interestingly, several gonococcal strains, such as strains MS11 and 1291, contain an extra NLS in the linker region (Fig. 1b), which, in the absence of a functional NalP, is never released into the medium of N. gonorrhoeae. Similarly, Haemophilus influenza IgA proteases (Poulsen et al., 1992) show a linker peptide sequence with one or two potential NLSs that remains attached to the β-domain. These observations underscore the potential importance of having these positively charged peptides on the cell surface.

Sequence analysis of the z-peptides of 13 gonococcal and 38 meningococcal strains revealed that they contain either one or four NLSs (Figs S4 and S5). In the meningococcal IgA proteases, all z-peptides that contain four NLSs are followed by an autoproteolytic cleavage site between the z-peptide and the linker domain. We observed that many of these z-peptides remained nevertheless associated as separate polypeptides with the bacterial cell surface (Fig. 3). We hypothesize that in these cases the z-peptides bind the negatively charged lipo-oligosaccharides via their N-terminal NLS, whilst the C-terminal NLS remain available to bind extracellular DNA or heparin. All meningococcal IgA proteases containing a single NLS are not followed by an autocatalytic processing site. Thus, these z-peptides remain covalently associated to the cell surface via the TD unless NalP is expressed. The latter mechanism offers the possibility to escape from the immune system when antibodies against the z-peptide are elicited and/or to escape from biofilms in order to colonize new host tissues. Importantly, we observed that the TD-linked z-peptides were removed from the cell surface by proteases present in serum (Fig. 5b). Therefore, this form of the protein...
presumably has a role only during the colonization of the nasopharynx, and not after crossing the epithelial layer and reaching the bloodstream.

In conclusion, we demonstrate for the first time that the z-peptide of IgA protease of N. meningitidis can remain associated with the bacterial cell surface either covalently linked via the TD or non-covalently associated as a separate polypeptide. In this location, it may exert new functions such as stimulating biofilm formation by binding extracellular DNA (Arenas 

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