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Lipidation of the autotransporter NalP of *Neisseria meningitidis* is required for its function in the release of cell-surface-exposed proteins

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Autotransporters of Gram-negative bacteria consist of an N-terminal signal sequence, a C-terminal translocator domain and the secreted passenger domain in between. The autotransporter NalP of *Neisseria meningitidis* includes a protease domain that facilitates the release of several immunogenic proteins from the cell surface into the extracellular milieu. Rather exceptionally among autotransporters, NalP is a lipoprotein. We investigated the role of lipidation in the biogenesis and function of the protein. To this end, the N-terminal cysteine, which is lipidated in the wild-type protein, was substituted by alanine. Like the wild-type protein, the mutant protein was secreted into the medium, demonstrating that lipidation is not required for biogenesis of the protein. However, the non-lipidated NalP variant had a drastically reduced capacity to cleave its substrate proteins from the cell surface, suggesting that the lipid moiety is important for function. Kinetic experiments demonstrated that the autocatalytic processing of the non-lipidated protein at the cell surface was much faster than that of the wild-type protein. Thus, the lipid moiety delays the release of NalP from the cell surface, thereby allowing it to release other surface-exposed proteins into the milieu.

INTRODUCTION

Proteins that are secreted by Gram-negative bacteria into the extracellular milieu have to cross a cell envelope consisting of the inner membrane, the periplasm, which harbours the peptidoglycan layer, and the outer membrane. The autotransporter pathway constitutes the most widespread secretion system among Gram-negative bacteria, and members of this class of proteins are often involved in virulence. Autotransporters consist of a passenger domain, which is the part of the protein that is actually secreted, an N-terminal signal sequence, which targets the protein to the Sec machinery for transport across the inner membrane, and a C-terminal translocator domain (TD), which is necessary for translocation of the passenger across the outer membrane (Benz & Schmidt, 2011; Dautin & Bernstein, 2007) (for an example, see Fig. 1a).

Proteins that are exported from the cytoplasm via the Sec machinery are targeted to the SecYEG translocon by a conserved N-terminal signal sequence. Although their amino acid sequences can be very diverse, signal sequences share a common structural organization. Usually, they are ~25 amino acid residues long and composed of a positively charged N terminus, followed by a hydrophobic central domain and a hydrophilic C terminus (Driessen & Nouwen, 2008). The latter domain contains the recognition site for leader peptidase, the enzyme that cleaves the signal sequence from the precursor after translocation across the inner membrane. In bacteria, at least two different leader peptidases coexist. Leader peptidase I cleaves the signal sequences from almost all precursors, the exception being the precursors of lipoproteins, which are cleaved by leader peptidase II (also known as Lsp). The signal sequences of lipoproteins contain a lipobox with the consensus sequence L(A/S)(G/A)C, which overlaps with the signal peptidase cleavage site (Okuda & Tokuda, 2011). The lipid molecules are attached to the cysteine in the lipobox, and this lipidated cysteine constitutes the N-terminal amino acid residue after processing by Lsp (Okuda & Tokuda, 2011).

The signal sequences of autotransporters in general display the characteristics of prototypical Sec-dependent signal sequences. However, a considerable number of autotransporters, including AIDA-I, EspP and Hbp from *Escherichia coli*, carry unusually long signal sequences of over 40 amino acid residues. The C-terminal halves of these signal sequences resemble normal signal sequences, but they contain an N-terminal extension with a unique and highly conserved sequence motif (Dautin & Bernstein, 2007; Henderson *et al.*, 1998). The conservation of this extension suggests that it has an important function, which, however, remains to be

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Abbreviations: EDDHA, ethylenediamine di-*o*-hydroxyphenylacetic acid; TD, translocator domain.

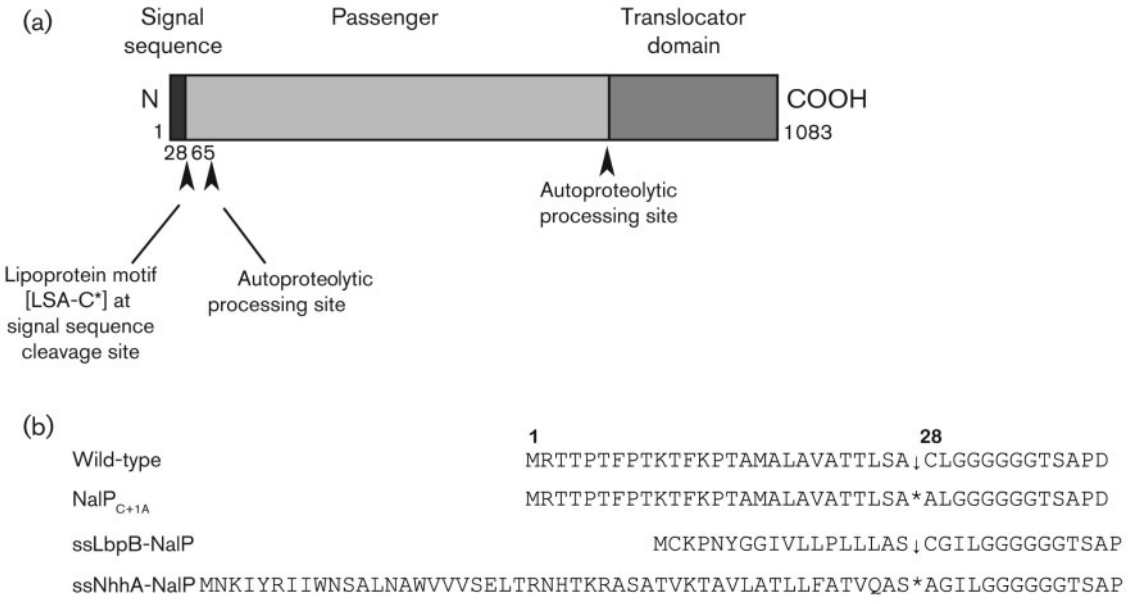

Fig. 1. Structural organization of the autotransporter NalP of *N. meningitidis* and signal sequence variants used in this study. (a) Structural organization of NalP. Various processing sites and amino acid residue positions are indicated. (b) Signal sequences of the NalP variants described in this study. The (predicted) cleavage sites for leader peptidases I and II are indicated by asterisks and arrows, respectively.

identified. It does not appear to be important for selection of the targeting route to the Sec machinery (Jong & Luirink, 2008). In one study, deletion of the N-terminal extension of the signal sequence of EspP did not interfere with efficient translocation of the protein across the inner membrane but led to its misfolding in the periplasm, thereby preventing subsequent secretion into the external medium (Szabady *et al.*, 2005). It was postulated that the extended signal sequence tethers the precursor to the periplasmic side of the inner membrane to prevent premature folding, which would block translocation across the outer membrane.

A small subset of autotransporters, including NalP of *Neisseria meningitidis* (Fig. 1a), contains a lipobox at the C-terminal end of the signal sequence. Lipidation has indeed been shown for NalP (van Ulsen *et al.*, 2003) as well as for SphB1 of *Bordetella pertussis* (Coutte *et al.*, 2003b), CapA of *Campylobacter jejuni* (Ashgar *et al.*, 2007) and AlpA of *Helicobacter pylori* (Odenbreit *et al.*, 1999). Why these autotransporters are lipidated is not completely understood. One possibility might be that the lipid moiety temporarily tethers the autotransporters to the inner membrane and, similar to the extended signal sequences, might be important for their proper biogenesis. Alternatively, it might be required for function.

In our lab, we are studying the structure, function and biogenesis of autotransporter NalP of *N. meningitidis*, a human pathogen that causes sepsis and meningitis. NalP is involved in the proteolytic release of other proteins from the cell surface (Roussel-Jazédé *et al.*, 2010; Serruto *et al.*, 2010; van Ulsen *et al.*, 2003). For example, the autotransporters IgA protease and App can be released from the cell surface, either by autocatalytic cleavage or via NalP-mediated cleavage. In the latter case, a larger version of the passenger, extended with a C-terminal domain known as the α-peptide, is released into the extracellular medium. It was postulated that NalP, being anchored in the outer membrane via its lipid moiety, can cleave these substrates closer to the cell surface, resulting in the release of the larger versions of these proteins (van Ulsen *et al.*, 2003). It should be noted, however, that the passenger domain of NalP itself was found to be efficiently released into the extracellular medium, because an N-terminal peptide including the lipid anchor was autocatalytically removed (van Ulsen *et al.*, 2003). Thus, anchoring of NalP to the cells via its lipid moiety is, at best, only temporary.

In this study, we investigated the role of the signal sequence and the lipid anchor of NalP. For this, the signal sequence of NalP was replaced by the N-terminally extended sequence of the trimeric autotransporter NhhA (van Ulsen *et al.*, 2001). We also substituted the cysteine that carries the lipid moiety by alanine via site-directed mutagenesis. The NalP variants were tested for secretion and for their activity in the release of neisserial surface proteins.

METHODS

Bacterial strains and growth conditions. All strains used are listed in Table 1. *E. coli* strains were grown at 37 °C in Luria–Bertani (LB)

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics*	Reference or source†
Strains		
E. coli		
DH5α		Lab collection
N. meningitidis		
HB-1	Unencapsulated derivative of H44/76	Bos & Tommassen (2005)
HB-1 *nalP*::*kan*	Insertion of Kan^R cassette in *nalP*	van Ulsen *et al.* (2003)
H44/76Sm^R	Spontaneous Sm^R, by a point mutation in the *rpsL* gene	This study
H44/76Δ*nalP*	Sm^R, deletion of the *nalP* gene without marker	This study
H44/76Δ*cap*Δ*nalP*	Sm^R, deletion of the capsule locus and the *nalP* gene without marker	This study
H44/76Δ*cap*Δ*nalP*Ω*300*	Sm^R, insertion of *nalP* in the *hrtA* locus, Erm^R	This study
H44/76Δ*cap*Δ*nalP*Ω*306*	Sm^R, insertion of gene encoding $NalP_{C+1A}$ in the *hrtA* locus, Erm^R	This study
H44/76Δ*cap*Δ*nalP*Ω*376*	Sm^R, insertion of gene encoding ssNhhA-NalP in the *hrtA* locus, Erm^R	This study
H44/76Δ*cap*Δ*nalP*Ω*377*	Sm^R, insertion of gene encoding ssLbpB-NalP in the *hrtA* locus, Erm^R	This study
Plasmids		
pCRII-TOPO	TA-cloning vector for PCR products, Amp^R, Kan^R	Invitrogen
pFLOB4300	Erm^R, Tet^R, contains the *rpsL* gene	Johnston & Cannon (1999)
pFLOB70′	Erm^R, Tet^R, contains the *rpsL* gene and DNA segment downstream of *nalP*	This study
pFLOB70′68′	Erm^R, Tet^R, contains DNA segments downstream and upstream of *nalP* with an *ermC′-rpsL* cassette inserted between	This study
pUC21	Amp^R	
pUC1970′	Amp^R, contains DNA segment downstream of *nalP*	This study
pUC70′68′	Amp^R, contains DNA segments upstream and downstream of *nalP*	This study
pRIT16845	Erm^R, contains the flanking regions of the capsule region with an *ermC′-rpsL* cassette inserted between	GSK
pRIT16840	Contains DNA segments up- and downstream of the capsule region	GSK
pPU100	Amp^R, encodes wild-type NhhA	van Ulsen *et al.* (2001)
pENLbpB	Cam^R, encodes LbpB	Roussel-Jazédé *et al.* (2010)
pPU300	Amp^R, encodes wild-type NalP	van Ulsen *et al.* (2003)
pCRII-mNalP	Amp^R, Kan^R, contains sequence coding mature NalP	This study
pCRII-ssNhhA-NalP	Amp^R, Kan^R, encodes ssNhhA-NalP	This study
pCRII-ssLbpB-NalP	Amp^R, Kan^R, encodes ssLbpB-NalP	This study
pCRT-hrtA	Amp^R, Kan^R, contains the complete *hrtA* region	This study
pEN300	Cam^R, encodes wild-type NalP	van Ulsen *et al.* (2003)
pEN306	Cam^R, encodes $NalP_{C+1A}$	This study
pEN376	Cam^R, encodes ssNhhA-NalP	This study
pEN377	Cam^R, encodes ssLbpB-NalP	This study
pCRT_hrtA_300	Amp^R, Kan^R, Erm^R, contains gene for wild-type NalP inserted in the *hrtA* region	This study
pCRT_hrtA_306	Amp^R, Kan^R, Erm^R, contains gene for $NalP_{C+1A}$ inserted in the *hrtA* region	This study
pCRT_hrtA_376	Amp^R, Kan^R, Erm^R, contains gene for ssNhhA-NalP inserted in the *hrtA* region	This study
pCRT_hrtA_377	Amp^R, Kan^R, Erm^R, contains gene for ssLbpB-NalP inserted in the *hrtA* region	This study

*Kan^R, Sm^R, Cam^R, Amp^R, Erm^R, Tet^R: resistance to kanamycin, streptomycin, chloramphenicol, ampicillin, erythromycin and tetracycline, respectively.
†GSK, GlaxoSmithKline Biologicals.

medium. *N. meningitidis* strains were grown on GC agar (Oxoid) supplemented with Vitox (Oxoid) at 37 °C in candle jars or in trypticase soy broth (TSB) (Gibco-BRL) at 37 °C with mild shaking. For plasmid maintenance, the following antibiotics were used: for *E. coli*, 100 μg ampicillin ml^{-1}, 50 μg kanamycin ml^{-1} or 25 μg chloramphenicol ml^{-1}; and for *N. meningitidis*, 5 μg chloramphenicol ml^{-1}, 375 μg streptomycin ml^{-1} or 7 μg erythromycin ml^{-1}. To impose iron limitation, TSB was supplemented with 20 μg ethylenediamine di-*o*-hydroxyphenylacetic acid (EDDHA) ml^{-1} (Sigma). When appropriate, IPTG was added to a final concentration of 1 mM to induce gene expression from the *lac* promoter.

Construction of strains and plasmids. Plasmids and primers used in this study are listed in Tables 1 and 2, respectively. PCRs were performed using High Fidelity *Taq* polymerase (Roche), and PCR products were cloned in pCRII-TOPO according to the manufacturer's instructions (Invitrogen). The resulting plasmids were sequenced to confirm the presence of the desired PCR fragments and the introduced mutations, as well as the absence of unwanted mutations.

To substitute an alanine residue for the cysteine in the NalP lipobox, a three-primer strategy was applied. Primers NalPCA and NalPstart

were used in a first PCR with pPU300 as the template. The PCR product was isolated and used as a megaprimer in a second PCR in combination with primer NalPseq6 and pPU300 as the template. The resulting PCR product was cloned in pCRII-TOPO, yielding pCRII-nalP$_{C+1A}$. The relevant fragment was excised from pCRII-nalP$_{C+1A}$ using *Nde*I and *Sal*I and used to substitute the corresponding fragment in pEN300, yielding pEN306.

To replace the signal sequence of NalP by that of NhhA or LbpB, a DNA fragment encoding NalP without its signal sequence and the cysteine at position +1 was obtained by PCR using primers mNalPBamHI and NalPEnd and pPU300 as the template. The PCR product was cloned into pCRII-TOPO, yielding pCRII-mNalP. A DNA fragment encoding the signal sequence of NhhA was obtained by PCR using primers ssNhhABamHI and LacApa and plasmid pPU100 as the template. A DNA fragment encoding the signal sequence of LbpB was obtained by PCR using primers ssLbpBBamHI and LacApa with plasmid pENLbpB as the template. The PCR products were cloned into pCRII-TOPO, from where they were excised by restriction with *Bam*HI and *Bst*EII and subsequently ligated into plasmid pCRII-mNalP, yielding pCRII-ssNhhA-NalP and pCRII-ssLbpB-NalP, respectively. To obtain the neisserial expression plasmids, the relevant fragments were cut from pCRII-ssNhhA-NalP and pCRII-ssLbpB-NalP with *Nde*I and *Sal*I and ligated into pEN300 digested with the same enzymes, yielding plasmids pEN376 and pEN377, respectively.

To replace the *nalP* gene on the chromosome by a cassette bearing *ermC′* and *rpsL*, a DNA fragment downstream of *nalP* was first amplified by PCR using primers 1970downfw_EcoRI and 1970downrev_PciI and chromosomal DNA of strain HB-1 as the template. This fragment was cloned into pFLOB4300 via *Eco*RI and *Pci*I, yielding plasmid pFLOB70′. The DNA upstream of *nalP* was amplified with primers 1968fw_HindIII and 1968rev_SacI and cloned into pFLOB70′ via *Sac*I and *Hin*dIII, yielding pFLOB70′68′. In addition, a plasmid was designed that carried DNA up- and downstream of *nalP* separated by a restriction site. To this end, the downstream fragment was amplified using primers 1970downfw_PstI and 1970downrev_XhoI and cloned into pUC21 via *Pst*I and *Xho*I, yielding pUC1970′. The upstream fragment was amplified with primers 1968fw_BamHI and 1968rev_PstI and cloned into pUC1970′ via *Pst*I and *Bam*HI, yielding pUC70′68′.

A markerless *nalP* knockout was obtained using a two-transformation-step procedure as previously described (van Dam & Bos, 2012). A piece of pFLOB70′68′ carrying the up- and downstream flanks of *nalP* separated by an *ermC′-rpsL* cassette was amplified by PCR using primers 1970downrev_dus and Rpsl_cassette_up. This PCR product was used to transform *N. meningitidis* strain H44/76 Sm^R, a spontaneous streptomycin-resistant derivative of strain H44/76 obtained as described previously (van Dam & Bos, 2012), selecting for erythromycin-resistant transformants. One of the erythromycin-resistant streptomycin-sensitive *nalP* knockouts obtained was then transformed with a fragment consisting of DNA up- and downstream of *nalP* that was amplified from pUC70′68′ by PCR using primers 1970downrev_dus and 1968fw_BamHI. Streptomycin-resistant transformants were picked and tested for erythromycin sensitivity. A proper markerless *nalP* knockout was confirmed by PCR, by sequencing over the scar region, and by Western blotting, and designated H44/76Δ*nalP*.

From H44/76Δ*nalP*, a markerless capsule-deficient mutant was subsequently isolated following a similar two-step procedure. A DNA fragment of pRIT16845 carrying the up- and downstream regions of the capsule locus separated by an *ermC′-rpsL* cassette was amplified by PCR using primers CapKOFLOBFWDUS45 and CapKOFLOBREV45. For the second transformation step, a DNA fragment consisting of the up- and downstream regions of the capsule region was amplified from pRIT16840 by PCR using primers CapKOFWDUS40 and CapKOREV40. A proper knockout was confirmed by colony blotting with capsule-specific monoclonal antibody 375 (Dade-Behring) and designated H44/76Δ*cap*Δ*nalP*.

For ectopic chromosomal expression of the various *nalP* constructs in *N. meningitidis*, the ORFs were placed in the *hrtA* locus of H44/76Δ*cap*Δ*nalP* under control of the *lac* promoter. A DNA fragment including the *hrtA* locus was obtained by PCR using primers HrtA_F1 and HrtA_R2 and chromosomal DNA of strain HB-1 as the template. The PCR products were cloned into pCRII-TOPO, yielding pCRT_hrtA. DNA fragments containing the *nalP* variants and the erythromycin-resistance gene were cut from plasmids pEN300, pEN306, pEN376 and pEN377 with *Aat*II and *Msc*I and subsequently blunted with T4 DNA polymerase. These fragments were ligated into pCRT_hrtA digested with *Bmg*BI, yielding pCRT_hrtA_300, pCRT_hrtA_306, pCRT_hrtA_376 and pCRT_hrtA_377, respectively. Subsequently, the *nalP* constructs were recombined into the chromosomal *hrtA* locus by selecting for erythromycin-resistant transformants.

Collection of cells and culture supernatants. Cells were harvested by centrifugation (4500 ***g***, 5 min) and resuspended in water to an OD_{550} of 10. The culture supernatants were centrifuged again (16 000 ***g***, 5 min) to remove residual cells. Then, the proteins were precipitated from the supernatant by adding ice-cold TCA to a final concentration of 5 % followed by incubation for at least 30 min at 4 °C and centrifugation for 20 min at 16 000 ***g***. The pellets were washed with 90 % acetone and dissolved in water. The supernatant fractions were 10-fold more concentrated than the cell samples. The cell and supernatant samples were mixed with an equal volume of 2-fold concentrated sample buffer and boiled for 10 min before electrophoresis.

SDS-PAGE and immunoblotting. Samples were loaded on gels with 10 % (w/v) polyacrylamide in the running gel. After SDS-PAGE, the proteins were blotted onto a 0.45 μm Protran filter (Schleicher and Schuell). Unspecific binding of antibodies to the filters was prevented by overnight incubation in PBS, pH 7.0, supplemented with 0.5 % non-fat dried milk (Nutricia) and 0.1 % Tween 20 (Merck). The antisera were diluted in the same buffer and applied for 1 h to the blots. After extensive washing, the blots were incubated with goat anti-rabbit or anti-mouse IgG antibodies conjugated to horseradish peroxidase (Biosource) at a dilution of 1 : 10 000 in the same buffer. Binding of antibodies was visualized by chemiluminescence using an ECL kit (Amersham).

Antisera. The polyclonal antisera against the full-length NalP, which recognizes only the passenger domain of the protein (van Ulsen *et al.*, 2003), the TD of NalP (Oomen *et al.*, 2004), LbpB (Pettersson *et al.*, 2006) and the protease domain of IgA protease of strain HF13 (Vidarsson *et al.*, 2005) were described previously. Antiserum against NhbA was generously provided by GlaxoSmithKline.

qRT-PCR. Quantitative real-time RT-PCR (qRT-PCR) was performed as described previously (Stork *et al.*, 2010). PCRs were performed in triplicate. The primer couples *nalP*_F_QPCR/*nalP*_R_QPCR and *rmpM*_Q_For/*rmpM*_Q_Rev (Table 2) were used to amplify the cDNAs of *nalP* and *rmpM*, respectively. The *rmpM* transcript was used to normalize all data.

RESULTS

Lipidation is not required for biogenesis of NalP

NalP possesses a signal sequence with a typical lipobox (Fig. 1a) and lipidation of the protein has been confirmed

Table 2. Primers used in this study

Primer	Sequence*	Restriction site
NalPCA	GCCTAAGGCGGCAGAAAGTGTTGTTGCA	
NalPstart	GGAATTCCATATGCGAACGACCCCAACCTTCCCTA	*Nde*I
NalPseq6	CGCCTGCGACTGTGATAATGCCT	
mNalPBamHI	GGGATCCTGGGCGGCGGCGGAGGCGGCA	*Bam*HI
NalPEnd	CAAGATCTCAGAACCGGTAGCCTACGCCGA	*Bgl*II
ssNhhABamHI	GGATCCCAGCACTTGCCTGAACCGTT	*Bam*HI
LacApa	CTTAATGGGCCCGCTAACAGCGCGAT	*Apa*I
ssLbpBBamHI	GGATCCCACAAGATGCCAAAAGTAAGGGCA	*Bam*HI
HrtA_F1	ATATAGGGCCCGAATTCGGACAGTTCTTCGA	
HrtA_R2	ATATACGATCGGAATTCGCCCGTCCGCTGA	
*nalP*_F_ QPCR	AACAGAGCGATTACGGCAAC	
*nalP*_R_QPCR	TATGTGTTGGGCTGAGCTTG	
*rmpM*_Q_For	AAACAACCTGGTCAGCAACG	
*rmpM*_Q_Rev	GCAACTTCGGCTTCACAAAC	
1970downfw_EcoRI	GCGCCTCGAATTCCCGGATTGCCATGCCTTATTTCGC	*Eco*RI
1970downrev_PciI	GCGGCGACATGTTTGGATGGTGCTGGTCATCTG	*Pci*I
1968fw_HindIII	GGGCGCAAGCTTCCAACATCACCAAAGTTTCGCTGGAAC	*Hin*dIII
1968rev_SacI	GCGGCCGAGCTCAACATCGACCTCGGTCATGACTGATTG	*Sac*I
1970downfw_PstI	GCGAATCCTGCAGCCGGATTGCCATGCCTTATTTCGC	*Pst*I
1970downrev_XhoI	GCAACGCTCGAGTTGGATGGTGCTGGTCATCTG	*Xho*I
1968fw_BamHI	GGCGGCGGATCCAACATCACCAAAGTTTCGCTGGAACTC	*Bam*HI
1968rev_PstI	GCCGCGCTGCAGAACATCGACCTCGGTCATGACTGATTG	*Pst*I
1970downrev_dus	ATGCCGTCTGAATTGGATGGTGCTGGTCATCTG	
Rpsl_cassette_up	GAGATCTGCAGGGCGATTAAGTTGGGTAACGCCAGGGT	
1970downrev_dus	ATGCCGTCTGAATTGGATGGTGCTGGTCATCTG	
1968fw_BamHI	GGCGGCGGATCCAACATCACCAAAGTTTCGCTGGAACTC	*Bam*HI
CapKOFLOBFWDUS45	ATGCCGTCTGAAGACCATGATTACGCCAAGCTC	
CapKOFLOBREV45	TATAGGGCGAATTGGGTACCG	
CapKOFWDUS40	ATGCCGTCTGAACGCCAAGCTCGAAATTAACC	
CapKOREV40	AAGTGCATCACGGCATCAG	

*Restriction sites present in the primers are underlined.

(van Ulsen *et al.*, 2003). To assess whether lipidation is essential for biogenesis of the protein and could exert a similar function to the typical extended signal sequences found in many other autotransporters, we constructed two NalP variants. First, we substituted the cysteine residue at the N terminus of the processed protein by alanine ($NalP_{C+1A}$ in Fig. 1b). The resulting NalP variant was predicted to contain a fairly normal signal sequence that could be processed by signal peptidase I according to an analysis by the SignalP 3.0 prediction server (Bendtsen *et al.*, 2004). Secondly, we replaced the entire NalP signal sequence including the lipobox and the N-terminal cysteine of the mature protein by the extended signal sequence of the meningococcal autotransporter NhhA (ssNhhA-NalP). As a control, the NalP signal sequence was also replaced by that of another cell-surface-exposed lipoprotein, i.e. the meningococcal lipoprotein LbpB (Pettersson *et al.*, 1998), yielding ssLbpB-NalP (Fig. 1b).

To express the different constructs in *N. meningitidis*, they were ectopically inserted into the chromosome. To this end, we created constructs containing the *nalP* variants under *lac* promoter control and an erythromycin-resistance cassette flanked by sequences of the *hrtA* locus, which was described to allow for a high rate of recombination (Claus *et al.*, 1998). These constructs were then inserted into the *hrtA* locus on the chromosome of H44/76Δ*cap*Δ*nalP* via homologous recombination. As a control, the wild-type *nalP* under control of the *lac* promoter was also inserted into the *hrtA* locus on the chromosome.

Expression of the recombinant genes in *N. meningitidis* was analysed by Western blot analysis using an anti-NalP antiserum that recognizes the passenger domain. As described previously for the wild-type strain (van Ulsen *et al.*, 2003), expression of wild-type *nalP* from the ectopic position resulted in secretion of the 70 kDa passenger into the medium (Fig. 2a). The secreted NalP passenger lacks the lipidated N-terminal moiety as a result of an autocatalytic cleavage after amino-acid residue 64 (Fig. 1a) by the NalP protease domain (van Ulsen *et al.*, 2003). Expression of the recombinant genes encoding $NalP_{C+1A}$ or ssLbpB-NalP resulted also in the release of an NalP passenger of similar size (Fig. 2a). It appears that secretion

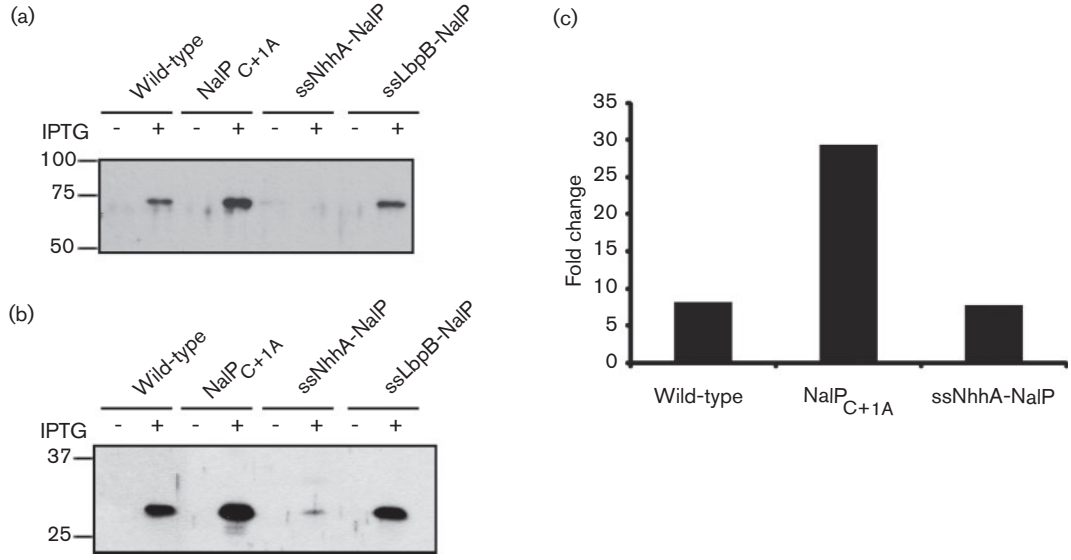

Fig. 2. Expression of the *nalP* variants under control of the *lac* promoter inserted in the *hrtA* locus of *N. meningitidis*. (a) Western blot of culture supernatants probed with anti-NalP antiserum. (b) Western blot of whole-cell lysates probed with antiserum recognizing the TD of NalP. (c) Expression of the *nalP* variants inserted in the *hrtA* locus relative to that of *nalP* in HB-1 as measured in qRT-PCR experiments. Expression levels were normalized to those of the *rmpM* gene. The experiment was performed once in triplicate.

is not impaired in these cases, although the substitutions may influence the expression levels, as considerably higher amounts of the $NalP_{C+1A}$ passenger were detected in the medium. By contrast, the passenger of ssNhhA-NalP was not detected in the culture supernatant (Fig. 2a), which could point to defects in the processing at the cell surface or in the expression, or it could result from degradation during passage through the cell envelope.

We then analysed whole-cell lysates of the different variants by Western blotting using an antiserum that recognizes the TD of NalP (Fig. 2b). Of the $NalP_{C+1A}$ and ssLbpB-NalP variants, only the ~32 kDa TD was detected, indicating that no secretion intermediates accumulated and that processing at the cell surface was efficient, similar to what was observed for wild-type NalP. In cells containing the *ssnhhA-nalP* fusion, only very low levels of the TD and no unprocessed full-length NalP were detected. Therefore, failure to detect the passenger of this variant in the culture supernatant (Fig. 2a) is not due to a processing defect in the outer membrane, but either to a low expression level or to degradation of the protein prior to its insertion into the outer membrane.

To determine whether the different amounts of NalP produced in the various strains could result from differences in gene expression, qRT-PCRs were performed. We compared expression levels of the ectopic copies of the *nalP* variants in strain H44/76Δ*cap*Δ*nalP* with that of the wild-type *nalP* in strain HB-1. All strains were grown for 5 h in the presence of 1 mM IPTG, after which total RNA was isolated and analysed. As a control, we analysed the expression of *rmpM*, which was similar between HB-1 and H44/76Δ*cap*Δ*nalP* expressing the ectopic *nalP* variants. Ectopic expression of $nalP_{C+1A}$ resulted in ~30-fold higher mRNA levels compared with wild-type chromosomal *nalP* and ~fourfold higher mRNA levels compared with ectopically expressed wild-type *nalP* (Fig. 2c). Thus, the higher levels of $NalP_{C+1A}$ protein observed on the blot (Fig. 2a) apparently result from the higher transcription of this recombinant gene. mRNA levels of ectopically expressed wild-type *nalP* and *ssnhhA-nalP* were both ~eightfold higher than those of *nalP* in the wild-type strain (Fig. 2c), indicating that the severely reduced amounts of the ssNhhA-NalP variant detected on blots (Fig. 2a, b) did not result from lower transcription. Thus, the presence of the extended signal sequence of NhhA in this variant may influence early steps of NalP biogenesis, resulting in protein degradation. An alternative explanation that cannot be excluded at this stage is that the efficiency of the translation of the mRNA is reduced.

Influence of lipidation of NalP on the processing of its substrates

NalP not only mediates its own processing but also that of other proteins at the bacterial cell surface, i.e. the autotransporters IgA protease, App and AusI (van Ulsen *et al.*, 2003, 2006), and the two surface-exposed lipoproteins LbpB and NhbA (also known as GNA2132) (Roussel-Jazédé *et al.*, 2010; Serruto *et al.*, 2010). To investigate whether lipidation of NalP is important for these functions, we first assessed the processing of the lipoproteins LbpB

and NhbA when expressed in the presence of either wild-type NalP or its variants. The *lbpB* gene is expressed under iron-limiting conditions and, therefore, the H44/76Δ*cap*Δ*nalP* derivatives carrying the ectopic *nalP* variants were grown in the presence of the iron chelator EDDHA. Expression of *nalP* was induced by the addition of IPTG and confirmed by Western blot analysis (results not shown). When wild-type NalP or ssLbpB-NalP was expressed, an approximately 100 kDa band representing LbpB was released from the cells (Fig. 3a, upper panel) and detected in the extracellular medium (Fig. 3a, lower panel). LbpB was not detected in the medium of cells expressing ssNhhA-NalP (Fig. 3a, lower panel), which could be due to the very low expression level of the protein (Fig. 2a, b). However, LbpB was also not detected in the supernatant of cells expressing $NalP_{C+1A}$, which is abundantly produced. Western-blot analysis of the whole-cell lysates showed that in this case LbpB remained associated with the cells (Fig. 3a, upper panel). In the case of NhbA, NalP-mediated cleavage was reported to result in the release of a 22 kDa fragment of the protein into the extracellular medium whilst a larger N-terminal fragment remained associated with the cells presumably via its lipid moiety (Serruto *et al.*, 2010). In accordance with the results obtained for LbpB, the NhbA protein was efficiently cleaved when cells expressed wild-type NalP (Fig. 3b, upper panel). However, the N-terminal fragment with an apparent molecular mass of approximately 55 kDa was not always detected in the cell lysates. When culture supernatants were analysed, small amounts of full-length NhbA were detected when NalP was not expressed, whilst both cleavage products of 55 and 22 kDa were detected when wild-type NalP was expressed (Fig. 4b, lower panel). Apparently, NalP not only cleaves NhbA to releases the 22 kDa fragment, but also releases the N-terminal 55 kDa fragment from the cell surface. NhbA was also released from the cells when ssLbpB-NalP was expressed, but much less efficiently upon expression of $NalP_{C+1A}$ (Fig. 3b, upper panel). As expected, no release of NhbA was observed in cells containing the poorly expressed *ssnhhA-nalP* construct. Therefore, we conclude that lipidation of NalP is required for cleavage of both LbpB and NhbA at the cell surface.

Next, we assessed the processing of the autotransporter IgA protease. Previously, we have shown that an approximately 120 kDa passenger domain of IgA protease is released after autocatalytic cleavage, whereas NalP-mediated cleavage results in the release of an approximately 160 kDa passenger

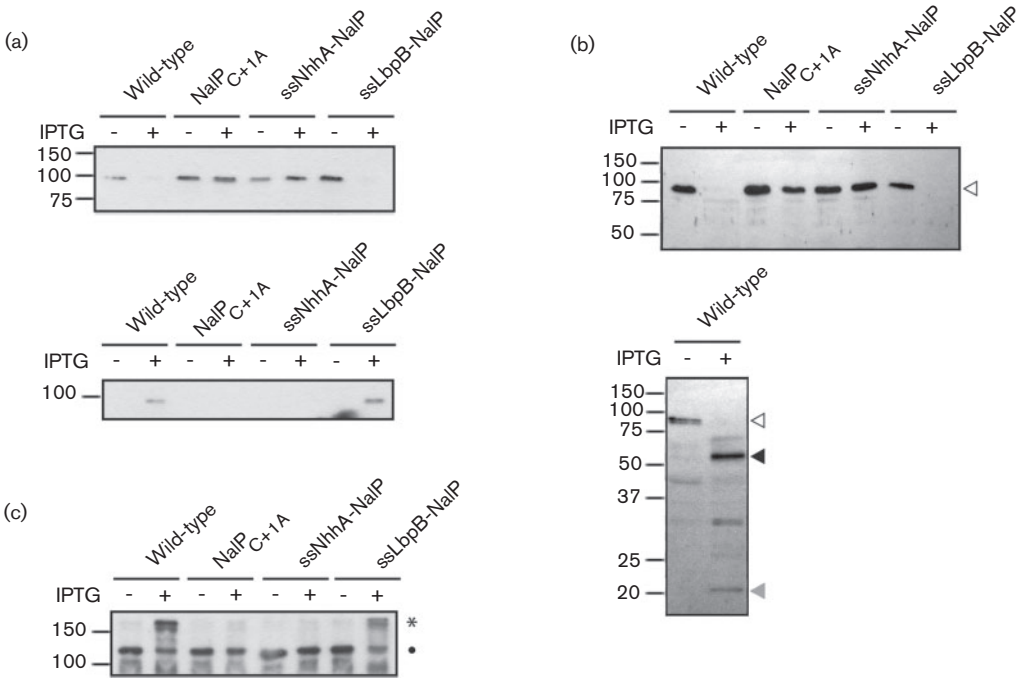

Fig. 3. NalP-mediated processing of cell-surface-exposed targets. Expression of the *nalP* variants was either induced with IPTG or not as indicated. (a) Western blots of whole-cell lysates (upper panel) and culture supernatants (lower panel) probed with anti-LbpB antiserum. (b) Western blots of whole-cell lysates (upper panel) and culture supernatants (lower panel) probed with anti-NhbA antiserum. In the upper panel, the full-length NhbA that remains associated with the cells is indicated with an open arrowhead. In the culture supernatants, small quantities of full-length NhbA (open arrowhead) are detected when NalP is not expressed, whereas degradation products of 55 kDa (black arrowhead) and 22 kDa (grey arrowhead) are detected when wild-type NalP is expressed. (c) Western blot of culture supernatants probed with antiserum that recognizes the protease domain of IgA protease. The asterisk and dot indicate secreted forms of IgA protease with and without α-peptide, respectively.

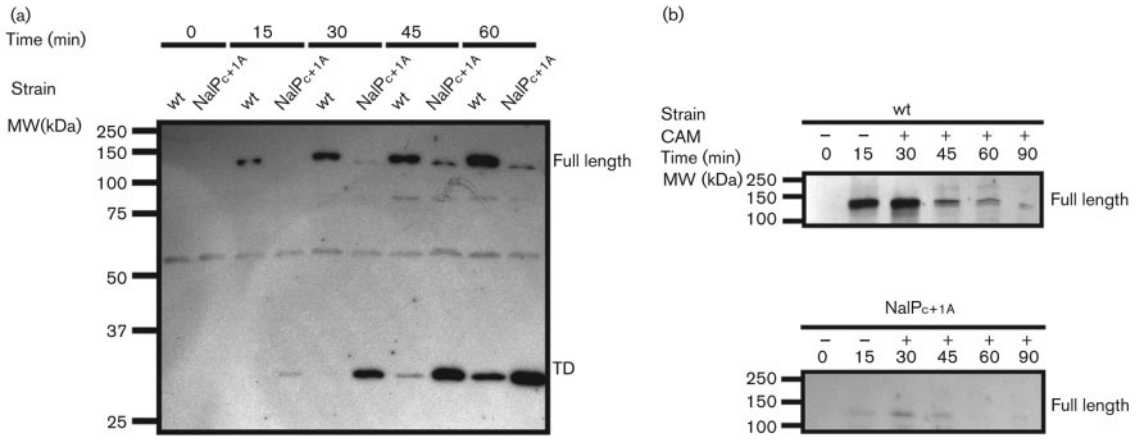

Fig. 4. Kinetics of autoprocessing of wild-type NalP and non-lipidated NalP at the cell surface. (a) Cells containing wild-type *nalP* or $nalP_{C+1A}$ both inserted into the *hrtA* locus were grown for 4 h in TSB to an OD_{550} of ~2.0. The cells were then diluted into fresh TSB supplemented with 1 mM IPTG to an OD_{550} of 0.25, and growth was continued for 1 h. Every 15 min, samples were taken to which 1 mM PMSF was added, and the samples were rapidly cooled on ice. The cells were harvested by centrifugation and whole-cell lysates were analysed by Western blotting using an antiserum directed against the TD of NalP. The positions of the full-length NalP and the TD are indicated on the right. (b) The same procedure was followed as in (a) except that 10 μg chloramphenicol (CAM) ml^{-1} was added 30 min after adding IPTG to inhibit further protein synthesis. Only the parts of the blots containing the full-length form of wild-type NalP (upper panel) or the $NalP_{C+1A}$ mutant protein (lower panel) are shown. wt, Wild-type.

that possesses an extra domain, the α-peptide (van Ulsen *et al.*, 2003). Western blot analysis showed that cells ectopically expressing wild-type *nalP* or *sslbpB-nalP* released both types of the passenger into the culture supernatant (Fig. 3c). In contrast, culture supernatants of cells that produced the non-lipidated variant $NalP_{C+1A}$ contained only the ~120 kDa passenger that results from autocatalytic cleavage. As expected, cells containing the poorly expressed *ssnhhA-nalP* chimeric gene also released only the 120 kDa form of IgA protease in the medium (Fig. 3c). Overall, our results demonstrate that the absence of the lipid moiety does not influence the proteolytic activity of NalP per se, as it does not interfere with the autocatalytic release of NalP from the cell surface. However, the absence of the lipid moiety largely abrogates the NalP-mediated release of other surface-exposed proteins.

Lipidation delays the release of NalP into the medium

The functional data described above could be explained if the lipid moiety of NalP retains the protein at the cell surface, allowing it to cleave other surface-exposed proteins. However, Western-blotting experiments suggested that the passenger domains of wild-type NalP and ssLbpB-NalP are released into the medium (Fig. 2a, b). Also, when we probed the blots containing the whole-cell lysates with an antiserum that recognizes the passenger domain of NalP, we failed to detect any cell-associated passenger on the blots (results not shown). Therefore, the lipidated NalPs are, at best, only temporarily retained at the cell surface. To assess this possibility, we determined the kinetics of release of the NalP passenger from cells ectopically expressing either wild-type NalP or $NalP_{C+1A}$. Cells were induced with IPTG and samples were taken every 15 min over 1 h. To prevent processing of NalP during sample preparation and analysis, a serine-protease inhibitor was immediately added to the samples. Western blot analysis of whole-cell lysates with an antiserum directed against the TD of NalP showed that $NalP_{C+1A}$ was rapidly processed as, at all time points, mostly the 32 kDa TD was detected in the cells (Fig. 4a). In contrast, the processing of wild-type NalP appeared much slower as large quantities of the full-length protein were detected in the lysates and the processed TD appeared only slowly over time (Fig. 4a). Thus, as hypothesized, the lipid moiety retards the release of NalP from the bacterial cell surface.

In the experiment illustrated in Fig. 4(a), the full-length form of wild-type NalP remained detectable in the cell lysates presumably because *de novo* protein synthesis continued during the entire experiment. To verify that the detected full-length form of the protein is released upon continued incubation, the experiment was repeated, but 30 min after induction of NalP synthesis with IPTG, chloramphenicol was added to inhibit further protein synthesis. The results showed that the full-length form of NalP that accumulates in the cells during the 30 min of protein synthesis is indeed released upon further incubation (Fig. 4b, upper panel). The full-length form of the mutant $NalP_{C+1A}$ protein again did not accumulate in substantial amounts during the 30 min of protein synthesis (Fig. 4b, lower panel).

DISCUSSION

The goal of our study was to investigate the role of the lipid moiety of NalP in the biogenesis and function of this autotransporter protease. One hypothesis was that the lipid moiety plays a role in the biogenesis of NalP by temporarily tethering the protein to the inner membrane. Such a function could influence the periplasmic folding of the protein, thereby affecting translocation across the outer membrane. In this model, the lipid moiety of NalP would have a similar function as has been suggested for the extended signal sequence of EspP (Szabady *et al.*, 2005). However, substitution of the cysteine in the lipobox, which is the residue where lipid modification occurs, by alanine did not impair secretion of the NalP passenger into the milieu. Moreover, we observed that the extended signal sequence of NhhA could not substitute the signal sequence and N-terminal cysteine of NalP, confirming that the lipid modification and extended signal sequences play different roles. The ssNhhA-NalP chimeric protein was poorly detected, which could be due to decreased gene expression or to increased proteolytic degradation. Our qRT-PCR data excluded gene-expression defects at the transcriptional level, and, although defects at the translational level cannot be excluded, we consider it more likely that the drastically lower amounts of NalP detected resulted from degradation of the protein, presumably as a consequence of a biogenesis defect. The exact function of the extended signal sequences in the biogenesis of autotransporters is under debate. Whilst the extended signal sequence of EspP appeared to influence periplasmic folding and thereby the translocation of EspP across the outer membrane (Szabady *et al.*, 2005), secretion of the *E. coli* autotransporter Pet seemed unaffected by the absence of the signal peptide extension (Leyton *et al.*, 2010). Our results seem to indicate that an extended signal sequence can influence biogenesis when fused to an autotransporter that is normally produced with a classical signal sequence. Apparently, the relationship between passenger and signal sequence is subtle and not yet fully understood. It will be interesting to generate additional constructs with swapped signal sequences and to study the effects on autotransporter biogenesis in further detail.

Whilst the lipid moiety is not required for the biogenesis of NalP, we considered the possibility that it is required for the protein's function in the release of other cell-surface-exposed proteins into the medium. As NalP itself was correctly secreted into the culture medium when expressed without its lipid moiety, lipidation is apparently not essential for autoproteolytic activity. However, the NalP-mediated release of other cell-surface-exposed proteins was severely hampered, demonstrating that lipidation of NalP is important for its function.

How could the lipid moiety promote the activity of NalP in processing cell-surface-exposed proteins? An obvious explanation would be that the lipid moiety tethers NalP to the cell surface thereby allowing it to cleave other proteins. However, also wild-type NalP is normally released into the medium (Fig. 2a, b) via N- and C-terminal autocatalytic cleavage events (van Ulsen *et al.*, 2003), indicating that the lipidated form of NalP can only be transiently present on the cell surface. To investigate this possibility, we studied the kinetics of the processing of wild-type and non-lipidated NalP. Indeed, we found that the full-length protein remains detectable for much longer in the cells when the protein is lipidated. Thus, due to the lipid moiety the protein remains longer in a position where it can cleave off other surface-exposed proteins.

Within the autotransporter family, lipoproteins constitute a rather small subset of proteins. Besides NalP, SphB1 of *B. pertussis* is another member of this subfamily that cleaves cell-surface-exposed molecules. Unlike NalP, SphB1 is not released to any significant extent into the extracellular medium; it remains associated with the cell surface, where it has been shown to target the filamentous haemagglutinin FHA (Coutte *et al.*, 2001). This proteolytic cleavage is essential for *B. pertussis* virulence (Coutte *et al.*, 2003a). Unlike the results presented here for NalP, site-directed mutagenesis targeting the N-terminal cysteine of the SphB1 passenger interfered with transport of the protein and resulted in its degradation. However, when the signal sequence of SphB1 was replaced by that of the autotransporter pertactin, which is not a lipoprotein, the SphB1 passenger was efficiently secreted and failed to cleave FHA (Coutte *et al.*, 2003b).

In conclusion, our results demonstrate that lipidation of NalP is not essential for the proper biogenesis of this autotransporter. However, the presence of the lipid moiety delays the autocatalytic release of NalP from the cell surface, thereby allowing the protein to cleave off other surface-exposed proteins.

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