Vaccination against *Neisseria meningitidis* Using Three Variants of the Lipoprotein GNA1870

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Abstract

Sepsis and meningitis caused by serogroup B meningococcus are devastating diseases of infants and young adults, which cannot yet be prevented by vaccination. By genome mining, we discovered GNA1870, a new surface-exposed lipoprotein of *Neisseria meningitidis* that induces high levels of bactericidal antibodies. The antigen is expressed by all strains of *N. meningitidis* tested. Sequencing of the gene in 71 strains representative of the genetic and geographic diversity of the *N. meningitidis* population, showed that the protein can be divided into three variants. Conservation within each variant ranges between 91.6 to 100%, while between the variants the conservation can be as low as 62.8%. The level of expression varies between strains, which can be classified as high, intermediate, and low expressors. Antibodies against a recombinant form of the protein elicit complement-mediated killing of the strains that carry the same variant and induce passive protection in the infant rat model. Bactericidal titers are highest against those strains expressing high yields of the protein; however, even the very low expressors are efficiently killed. The novel antigen is a top candidate for the development of a new vaccine against meningococcus.

Key words: Neisseria • antigen • vaccine • gene variability

Introduction

Neisseria meningitidis is a gram negative, encapsulated bacterium which at any time colonizes the upper respiratory tract of ~10% of human population. Colonizing strains belong to hypervirulent lineages and carriage strains, which are frequently or rarely associated with disease, respectively. During nonepidemic periods approximately once in every 10,000 colonized people or once in 100,000 population, the bacterium enters the blood stream where it multiplies and gives sepsis. During epidemic periods, the attack rates can be much higher. From the blood stream the bacterium may cross the blood-brain barrier and cause meningitis. Both diseases are devastating and can kill 5–15% of the affected children and young adults within hours, despite the availability of effective antibiotics (1). Up to 25% of those who survive are left with permanent sequelae, which may include amputation of limbs, mental retardation, and hearing loss (2, 3). The bacteria have been classified in serogroups based on the chemical composition of the polysaccharide capsule (4). Although 13 chemically different serogroups have been described, only serogroups A, B, C, Y, and W-135, and to a very minor extent X and Z, have been associated with disease (5, 6). In addition to the capsular serogroup, most of the meningococcal strains isolated from invasive disease, have been classified by multilocus enzyme electrophoresis (MLEE) into a small number of hypervirulent lineages: Electrophoretic Types ET-37, ET-5, cluster A4, lineage 3, subgroups I, III, and IV-1 (7, 8). Recently, a new sequence-based classification, multilocus sequence typing (MLST) has been introduced, which classifies the above strains mainly into Sequence Types ST11, ST32, ST8, ST41, ST1, ST5, ST4, respectively (9).

V. Masignani and M. Comanducci contributed equally to this work.

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Carrier strains have a more diverse clustering, and a wider range of genotypes.

Prevention of disease can effectively be accomplished by vaccination. Immunization was made possible in 1969 when it was discovered that protection from disease correlates with the presence in the serum of antibodies able to induce complement-mediated killing of bacteria, and that purified capsular polysaccharide was able to induce them. Tetravalent vaccines against serogroups A, C, W-135, and Y have been available since 1984 (10–12). Although effective in adults, polysaccharide vaccines are less efficacious in infants and young children and do not induce immunological memory, so that they have not been used for universal vaccination. Recently, conjugate vaccines against serogroup C have been introduced in the UK, Ireland, and Canada (13, 14). These are immunogenic in infants and children, induce immunological memory and show overall efficacy of more than 90%. Tetravalent conjugate vaccines against serogroups A, C, W-135, and Y are presently in clinical development (15). As a consequence, during the next 4–6 yr we expect to be able to prevent diseases caused by serogroups A, C, W-135, and Y in all age groups. Unfortunately, the conjugate approach cannot be easily applied to serogroup B because the capsular polysaccharide is a polymer of $\alpha(2\rightarrow8)$*N*-acetyl neuraminic acid or polysialic acid, a molecule which is identical to a widely distributed human carbohydrate. Being the serogroup B polysaccharide identical to a self-antigen, man is immunologically tolerant to it and therefore fails to induce an effective immune response. On the other hand, an immune response against it would risk to induce autoimmunity (16, 17). For this reason, there are no licensed vaccines available in the USA and Europe for the prevention of serogroup B *N. meningitidis* disease, which is responsible for 32% of all meningococcal disease in the United States, for 45 to 80% of the cases in Europe and for more than 50% of the cases in the rest of the world, with the exception of Sub-Saharan Africa where serogroup A is responsible for 90% of the cases (18, 19). To develop a vaccine against serogroup B, surface-exposed proteins contained in outer membrane vesicles (OMVs)* have been used (20, 21). These vaccines elicit serum bactericidal antibody responses and protect against meningococcal disease (22, 23). However, while they induce complement-mediated bactericidal antibodies against the homologous strain, they fail to induce bactericidal antibodies against heterologous strains (24). For this reason their use has been limited only to Central and South America.

In the year 2000 our laboratory published the use of the genomic sequence of MC58 to discover novel antigens capable of inducing protection against serogroup B *N. meningitidis* (25). Here we describe a novel antigen which was discovered by mining the bacterial genome and that is very effective in inducing bactericidal antibodies. This antigen is a very good candidate for inclusion in universal vaccines against *N. meningitidis*.

Abbreviation used in this paper: OMV, outer membrane vesicle.

Materials and Methods

Strains. *Escherichia coli* DH5α and BL21(DE_3) were used as cloning strain and expression host, respectively, and used as recommended by the manufacturer (Invitrogen).

N. meningitidis strains used were described by Comanducci et al. (26). Selection was made to represent the meningococcal diversity both in terms of genotype and geographic distribution (they derive from 19 different countries and 73% belong to serogroup B). 32/71 (45%) were isolated during the last five years. Most data in this paper refer to strains: MC58, 961–5945, M1239, 67/00, NZ394/98, H44/76, Cu385, (26), and 4243 (27). The NZ394/98 strain was originally described by Diana Martin as NZ98/254 (Institute of Environmental Science & Research Limited, Kenepuru Science Centre, Porirua, New Zealand).

GNA1870 Cloning, Expression, and Purification in E. coli. *gna1870* genes were amplified by PCR from the genome of *N. meningitidis* MC58, 961–5945 and M1239 strains, respectively. Forward and reverse primers were designed in order to amplify the *gna1870* coding sequence devoid of the sequence coding for the putative leader peptide. M1239 and 961–5945 variants were found not to be expressible in *E. coli*. Therefore, they were expressed by adding to the NH_2-terminal the sequence GPDSDRLQQRRG that is present in the gonococcus protein, but absent in the meningococcus counterpart. Oligonucleotides used for the amplification are reported as follows: strain MC58 (For1: CGCGGATCCCATATGGTCGCCGCCGACATCG; Rev1: CCCGCTCGAGTTGCTTGGCGGCAAGGC). strain 961–5945(For2: CGCGGATCCCATATG*GGCCCTGATTCTGACCGCCTGCAGCAGCGGAGG*__GTCGCCGCCGACATCGG__; Rev2:CCCGCTCGAGCTGTTTGCCGGCGATGCC); strain M1239 (For 2; Rev3:GCCCAAGCTTCTGTTTGCCGGCGATGCC).

Restriction sites, corresponding to NdeI for the forward primers and XhoI for the reverse primers, are underlined. In the case of M1239 strain the restriction site used for the reverse primer is Hind III. For the 961–5945 and M1239 forward primers, the gonococcus sequence moiety is reported in italics, whereas the meningococcal GNA1870 matching sequences are reported in bold.

PCR conditions in the case of primer combination For1/Rev1 were: denaturation at 94°C for 30 s, annealing at 57°C for 30 s, elongation at 68°C for 1 min (5 cycles), denaturation at 94°C for 30 s, annealing at 68°C for 30 s, elongation at 68°C for 1 min (30 cycles). In the case of primer combinations: For2/Rev2, For3/Rev2, and For3/Rev3: 94°C for 30 s, 56°C for 30 s, 68°C for 1 min (5 cycles), 94°C for 30 s, 71°C for 30 s, 68°C for 1 min (30 cycles).

gna1870 full-length gene was amplified from the MC58 genome using the following primers: f-lFor (CGCGGATCCCATATGAATCGAACTGCCTTCTGCTGCC) and f-lRev (CCCGCTCGAGTTATTGCTTGGCGGCAAGGC) and the following conditions: 94°C for 30 s, 58°C for 30 s, 72°C for 1 min (30 cycles).

PCR were performed on ~10 ng of chromosomal DNA using High Fidelity Taq DNA Polymerase (Invitrogen). The pCR products were digested with NdeI and XhoI and cloned into the NdeI/XhoI sites of the pET-21b+ expression vector (Novagen).

Recombinant proteins were expressed as His-tag fusions and purified by MCAC (Metal Chelating Affinity Chromatography), as described previously (25).

gna1870 Gene Sequencing. *gna1870* gene was amplified using primers external to the coding sequence (A1: GACCTGCCTCATTGATG and B2: CGGTAAATTATCGTGTTCGGACGGC). About 10 ng of chromosomal DNA were used as

template for the amplification. PCR conditions were: 30 cycles, 94°C for 40 s, 58°C for 40 s, 68°C for 40 s. PCR fragment were purified by the QIAGEN QIAquick PCR Purification Kit, and submitted to sequence analysis, which was performed using an ABI 377 Automatic Sequencer. The sequence was performed using primers A1, B2, 22 (CAAATCGAAGTGGACGGGCAG), and 32 (TGTTCGATTTTGCCGTTTCCCTG). Sequence analysis was performed using Editview, GeneJockey, MacBoxshade.

Computer Analysis. The GCG Wisconsin Package suite (version 10.0) was used for computer analysis of gene and protein sequences. The PSORT program (28) was used for localization prediction. For secondary structure analysis, the PredictProtein software available on the web at http://cubic.bioc.columbia.edu/predictprotein/ was applied. The PSI-BLAST algorithm was used for homology searches (http://www.ncbi.nlm.nih.gov/blast) using the nonredundant protein database. The prediction of the Fur box was performed with FindPatterns (GCG) starting from the *E. coli* consensus (29) and allowing a maximum of nine mismatches. Search for lipoproteins with a glycine-rich region has been performed on 22 complete genomic sequences retrieved at the NCBI site (http://www.ncbi.nlm.nih.gov/PMGifs/Genomes/micr.html) using FindPatterns. The complete genome sequence of *Neisseria gonorrhoeae* strain FA1090 is available at the site http://dna1.chem.ou.edu/gono.html.

The dendrograms were obtained starting from the multiple sequence alignment of GNA1870 protein sequences (PileUP) using the Protein Sequence Parsimony Method (ProtPars), a program available within the Phylogeny Inference Package (Phylip) (Felsenstein, J. 1993, program distributed by the author), and confirmed by the GCG program Distances, using the Kimura and Jukes-Cantor algorithms.

gna1870 and siaD Isogenic Mutants. Isogenic knockout mutants in which the *gna1870* gene was truncated and replaced with an erythromycin antibiotic cassette, was prepared by transforming strains MC58, 961–5945 and M1239 with the plasmid pBSΔ*gna1870*ERM. This plasmid contains the erythromycin resistance gene within the *gna1870* upstream and downstream flanking regions of 500 bp. These regions were amplified from MC58 genome using the following oligonucleotides UFor-GCTCTAGACCAGCCAGGCGCATAC (Xba1 site underlined); URev TCCCCCGGGGACGGCATTTTGTTTACAGG (Sma1 site underlined); DFor TCCCCCGGGCGCCAAGCAATAACCATTG (Sma1 site underlined) and DRev CCCGCTCGAGCAGCGTATCGAACCATGC (Xho1 site underlined). A capsule-deficient mutant was generated using the same approach. The *siaD* gene was deleted and replaced with *ermC* using the plasmid pBSΔ*Cap*ERM. The upstream and downstream flanking regions of 1,000 bp and 1,056 bp, respectively, were amplified from MC58 genome using the following primers: UCapFor GCTCTAGATTCTTTCCCAAGAACTCTC (Xba1 site underlined); UcapRev TCCCCCGGGCCCGTATCATCCACCAC (Sma1 site underlined); DCapFor TCCCCCGGGATCCACGCAAATACCCC (*Sma*1 site underlined) and DCapRev CCCGCTCGAGATATAAGTGGAAGACGGA (Xho1 site underlined). Amplified fragments were cloned into pBluescript and transformed into naturally competent *N. meningitidis* strain MC58. The mixture was spotted onto a GC agar plate, incubated for 6 h at 37°C, 5% CO_2, then diluted in PBS and spread on GC agar plates containing 5 μg/ml erythromycin. The deletion of the *gna1870* gene in the MC58Δ*gna1870*, 961–5945Δ*gna1870*, and M1239Δ*gna1870* strains was confirmed by PCR; lack of GNA1870 expression was confirmed by Western blot analysis. The deletion of the *siaD* gene and the lack of capsule expression in the MC58Δ*siaD* strain were confirmed by PCR and FACS®, respectively.

Expression of GNA1870 during Growth. MC58 strain was grown at 37°C with 5% CO_2 in GC medium at stationary phase. Samples were collected during growth (OD_{620} of 0.05–0.9). MC58Δ*gna1870* was grown until the OD_{620} of 0.5. Bacterial cells were collected by centrifugation, washed once with PBS, and resuspended in various volumes of PBS in order to standardize the OD values. Culture supernatant was filtered using a 0.2 μm filter and 1 ml precipitated by the addition of 250 μl of 50% trichloroacetic acid (TCA). The sample was incubated on ice for 2 h, centrifuged for 40 min at 4°C and the pellet washed with 70% ice cold ethanol, and resuspended in PBS. 3 μl of each sample (corresponding to an OD_{620} of 0.03) was then loaded on a 12% polyacrylamide gels and electrotransferred onto nitrocellulose membranes. Western blot analysis was performed according to standard procedures (30), using anti-GNA1870 polyclonal antibodies at a 1:1,000 dilution, followed by a 1:2,000 dilution of HPR-labeled anti-human IgG (Sigma-Aldrich). Scanning was performed using a LabScan (Amersham Biosciences) and Imagemaster software (Amersham Biosciences).

[^{3}H]-palmitic Acid Labeling of E. coli and N. meningitidis. Palmitate incorporation of recombinant *E. coli* BL21(DE3) strain carrying the full-length *gna1870* gene was confirmed as described by Jennings et al. (31).

Meningococcal strains MC58 and MC58Δ*gna1870* were grown in GC medium and labeled with [9,10-^{3}H]-palmitic acid (Amersham Biosciences). Cells from 5 ml culture were lysed by boiling for 10 min and centrifuged at 13,000 rpm. The supernatants were precipitated with TCA and washed twice with cold acetone. Proteins were suspended in 50 μl of 1.0% SDS and 15 μl analyzed by SDS-PAGE, stained with Coomassie brilliant blue, fixed, and soaked for 15 min in Amplify solution (Amersham Biosciences). Gels were exposed to Hyperfilm MP (Amersham Biosciences) at –80°C for 3 d.

Binding Assay to Human Transferrin. Recombinant GNA1870, human transferrin hTF (Sigma T-4132) and the mix of the two (final concentration of 7 μM) were dialysed O/N in PBS at 4°C. After dialysis 20 μl of each protein and the mixture of them were loaded on a HPLC Superdex 200 PC 3.2/30 gel filtration column (Amersham Biosciences) using PBS as running buffer (32). Blue Dextran 2000 and the molecular weight standards ribonuclease A, chymotrypsinogen A, ovalbumin, bovine serum albumin (Amersham Biosciences) were used to calibrate the column. Gel filtration was performed using a Smart system with a flow rate of 0.04 ml/min and the eluted material monitored at 214 and 280 nm (the GNA1870 retention volume was 1.68 ml and 1.47 ml for htf). Fractions of 40 μl were collected and analyzed by SDS-PAGE. The MC58 recombinant transferrin-binding protein 2 (Tbp2) was used as positive control.

Mice Immunizations. To prepare antisera, 20 μg of variant 1, variant 2, and variant 3 GNA1870 recombinant proteins were used to immunize 6-wk-old CD1 female mice (Charles River Laboratories). Four to six mice per group were used. The recombinant proteins were given intraperitoneally, together with CFA for the first dose and IFA for the second (day 21) and third (day 35) booster doses. The same immunization schedule were performed using Aluminium hydroxide (3 mg/ml) instead of Freund's adjuvant. Blood samples for analysis were taken on day 49.

FACS® Analysis. The ability of polyclonal anti-GNA1870 antisera to bind to the surface of live meningococci was determined using FACScan™ flow cytometer on the MC58, MC58Δ*siaD* (nonencapsulated) and MC58Δ*gna1870*, on 961–

5945 and 961–5945Δ*gna1870*, on M1239 and M1239Δ*gna1870*, on NZ394/98 and 67/00 strains. Antibody binding was detected using a secondary antibody anti–mouse (whole molecule) FITC-conjugated (Sigma-Aldrich). The positive control included SEAM 3, a mAb specific for the meningococcus B capsular polysaccharide (33). The negative control consisted of a mouse polyclonal antiserum against the cytoplasmic protein NMB1380 (34).

Complement-mediated Bactericidal Activity. Serum bactericidal activity against *N. meningitidis* strains was evaluated as described previously (25, 35), with pooled baby rabbit serum (CedarLane) used as complement source. A human serum from an healthy adult (with no intrinsic bactericidal activity when tested at a final concentration of 25 or 50%) was also used as complement source. Serum bactericidal titers were defined as the serum dilution resulting in 50% decrease in CFU per ml after 60 min incubation of bacteria with reaction mixture, compared with control CFU per ml at time 0. Typically, bacteria incubated with the negative control antibody in the presence of complement showed a 150 to 200% increase in CFU/ml during the 60 min incubation.

Western Blot Analysis on Human Sera. Purified GNA1870 (1 μg/lane) was loaded onto 12.5% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. The bound protein was detected with 1:200 dilution of convalescent or normal sera, followed by a 1:2,000 dilution of HPR-labeled anti–human IgG (Sigma-Aldrich).

Rat Protection. 5-d-old infant rats were pretreated intraperitoneally with anti-GNA1870 antisera or with anti-PorA monoclonal antibody at time 0 and challenged 2 h later intraperitoneally with 1.9×10^3 CFU/rat of MenC 4243 (OAc-positive). Quantitative blood cultures were obtained 18 h later. Bacterial counts in the blood cultures were obtained by plating 100, 10, and 1 μl (10 μl of a 1:10 dilution) of blood on chocolate agar plates.

Results

GNA1870 Gene and Protein Features. Genome-derived Neisseria Antigen 1870 (GNA1870) was selected while mining the still incomplete genome of the serogroup B strain MC58, because it had the typical signature of a surface-exposed lipoprotein. This was characterized by a signal peptide with a lipo-box motif of the type –Leu-X-X-Cys, where the Cysteine was followed by a Serine, an amino acid generally associated with outer membrane localization of lipoproteins (36). The gene was annotated as *nmb*1870 and *nma*0586 in the genome sequences of MenB MC58 and MenA Z2491 published by The Institute for Genomic Research (TIGR) and Sanger Center, respectively (37, 38). Interestingly, the electronic annotation of TIGR and Sanger assigned the gene start to ATG sequences located 138 and 18 base pairs upstream from the GTG that we propose as the real start codon. This new position is consistent with the presence of a correctly spaced ribosome-binding site, as well as with the prediction of the lipoprotein signature (Fig. 1).

According to our analysis, *gna*1870 is a monocistronic gene and is located 157 bases downstream the stop codon of the fructose-bisphosphate aldolase gene *nmb*1869 (Fig. 1). In MenA Z2491 the overall organization is similar, however, 31 base pairs upstream from the GTG starting codon there is an insertion of 186 nucleotides that are homologous to an internal repeat region of IS1106, and are flanked by two 16 base pairs inverted repeats. A putative ribosome binding site and a putative Fur box are present 8 and 35 bp, respectively, upstream from the GTG starting codon. Putative promoter sequences were also detected. The mature protein is predicted to be a lipoprotein with a molecular weight of 26,964 Daltons and an isoelectric point of 7.96 in MC58, and is characterized by the presence of four Glycines downstream of the lipo-box motif. Secondary structure prediction analysis indicates that GNA1870 is a globular protein mostly composed of β sheets. No homologous proteins were found by searching existing nonredundant prokaryotic and eukaryotic protein databases maintained at the NCBI site, including the human genome, suggesting that this protein is specific for *Neisseria*. However, a domain with some homology (28% identity over 146 amino acids) was found with the COOH-terminal portion of the transferrin-binding protein

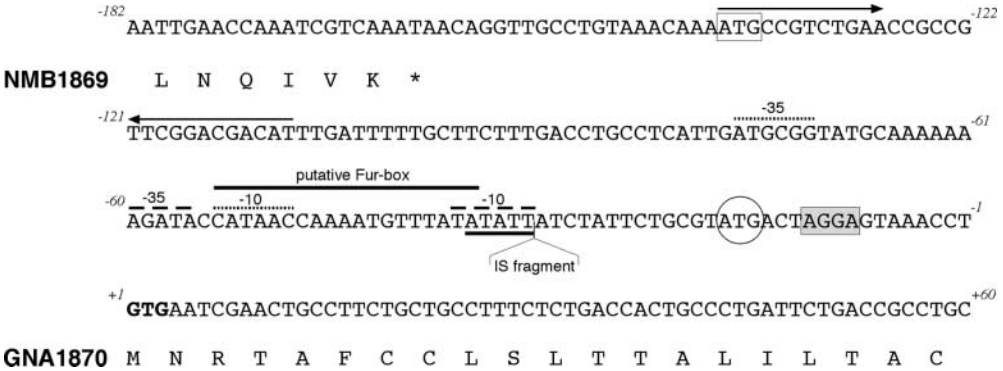

Figure 1. Nucleotide sequence of the region upstream gene *gna1870* in MenB strain MC58. The DNA and deduced amino acid translation of the GNA1870 predicted lipoprotein signal peptide are also shown. The translation initiation codon GTG is positioned at nucleotide +1. The putative ribosome binding site (RBS) is shaded and two possible –10 and –35 promoter elements are indicated by dashed lines and dots, respectively. A putative Fur box is indicated (the sequence CATAACCAAAATGTTTATA matches the *E. coli* Fur box consensus GATAATGATAATCATTATC in 11 over 19 bases). Horizontal arrows indicate two inverted repeats containing a DNA uptake sequence. The start codons attributed by TIGR and Sanger are indicated by a box and a circle, respectively. The pentanucleotide ATATT used as site of insertion for the IS fragment in MenA strain Z2491 is underlined. In MenA, the presence of the IS fragment creates novel –10 and –35 elements. In Gonococcus, the whole region is conserved with that of MenB, however, the insertion of a single base (G) after position 36 causes a frame-shift, which results in the loss of the lipo-box motif. The correct frame is reestablished with a further eight-base insertion after position 73.

TfbA of *Actinobacillus pleuropneumoniae* (39). This amino acid homology does not seem to reflect a functional homology since the recombinant protein failed to bind human transferrin in vitro (data not depicted).

Protein Expression and Lipidation. To produce a recombinant GNA1870, either the full-length gene or the region coding for amino acids 8–255 were amplified from *N. meningitidis* strain MC58 by PCR and cloned in the expression vector pET21b+. The recombinant COOH-terminal His-tagged fusion protein was purified and used to immunize mice to obtain antisera. When the full-length clone was grown in the presence of [9,10-^{3}H]-palmitic acid, a radioactive band was detected at the expected molecular weight, confirming that *E. coli* recognizes the lipoprotein motif and adds a lipid tail to the recombinant protein (data not depicted). A radioactive band of the appropriate molecular weight was also detected in *N. meningitidis* grown in the presence of [9,10-^{3}H]-palmitic acid (data not depicted). We conclude that GNA1870 is a lipoprotein both in *N. meningitidis* and in *E. coli*. The lipoprotein is surface-exposed and expressed at different levels by different strains.

Immune sera raised against the recombinant protein were used to detect the lipoprotein in *N. meningitidis* MC58 by Western blotting and FACS®. As shown in Fig. 2, a protein of ~29.5 kD was detected in the total cell extracts of *N. meningitidis*. Scanning of the Western blot in Fig. 2 A showed that the amount of the protein in the whole cell lysate increased approximately two times during the growth curve, while the optical density of the culture increased from 0.05 to 0.9 OD_{620nm}. Interestingly, a band of the same size was also detected in the culture supernatant. The protein was not detected in the supernatant of the freshly inoculated culture (OD_{620nm} of 0.05), and increased approximately four times during the growth from 0.1 to 0.9 OD_{620nm} (Fig. 2 B, insert 1). The genuine nature of the supernatant was confirmed by testing the same samples for the absence of membrane blebs and of cytoplasmic proteins. As shown in Fig. 2 B, insert 2, the absence in the supernatant preparations of PorA, known to be a major component of membrane blebs, rules out a possible contamination with membrane blebs, while the absence in the supernatant of the cytoplasmic protein NMB1380 confirmed that the supernatant samples do not result from cell lysis (Fig. 2 B, insert 3). The MC58*Δgna1870* strain, where the gene coding for the protein GNA1870 had been deleted, shows no protein either in the whole cell lysate or in the culture supernatant (KO lane, in Fig. 2, A and B). The protein was detected by Western blotting in outer membrane vesicles vaccines prepared by the Norwegian Institute of Public Health (NIPH), confirming that the protein segregates with the membrane fractions of *N. meningitidis* (Fig. 2 C). Interestingly, sera from mice immunized with the NIPH vaccine did not recognize the recombinant GNA1870 in Western blotting (data not shown), suggesting that the protein is not immunogenic in these preparations.

FACS® analysis confirmed that the protein is surface-exposed and accessible to antibodies both in encapsulated and nonencapsulated *N. meningitidis* strains (Fig. 2 D). Western blotting analysis of 43 strains showed that the protein was expressed by all strains tested. However, as shown in Fig. 3, the levels of expression varied considerably from strain to

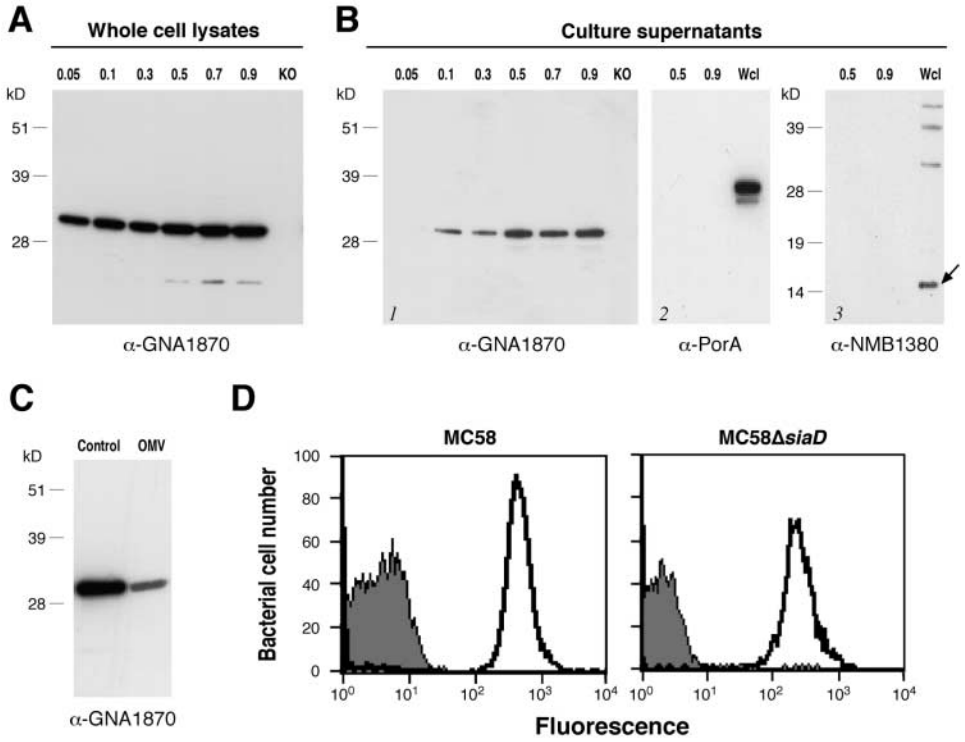

Figure 2. Expression of GNA-1870 in strain MC58. (A and insert 1 of B) Western blots of whole cell lysates and culture supernatants, respectively. Cultures were harvested at different times during the growth curve and probed with the anti-GNA1870 serum. (B, inserts 2 and 3) Culture supernatants probed with anti-PorA and anti-NMB1380 antisera, respectively. The OD_{620} of the culture is indicated above each lane. KO indicates a *gna*1870 knock-out mutant of MC58. Wcl stands for whole cell lysate used as control. (C) Outer membrane vesicle (OMV) vaccine from the Norwegian Institute of Public Health and recombinant GNA1870 used as positive control probed with anti-GNA1870 serum. (D) FACS® analysis of encapsulated MC58 and of its nonencapsulated isogenic mutant using the serum against GNA1870. Shaded profile is the negative control obtained by reaction with the preimmune serum. Non-shaded profile shows reaction with immune sera.

strain. The strains tested could be broadly classified as high, intermediate, and low expressors. Scanning of the Western blots showed that the difference in expression between high and intermediate, intermediate and low or high and low could be two-, five-, and ninefold, respectively. As shown in Table I, of the 43 strains tested, 25 (58%) expressed high levels of GNA1870, 11 (25.5%) expressed intermediate levels, and 7 (16.5%) expressed low levels. Most of the strains from hypervirulent lineages (ET-5, lineage 3, and ET-37) expressed high levels of the protein, with the exception of A4 isolates where two strains expressed intermediate levels and two expressed low levels. Interestingly, the protein was expressed at high level by strains that have been classically used as OMV vaccine strains (H44/76 and Cu385). No obvious genetic patterns were found to predict the amount of protein expressed by each strain. Even the presence of the IS element in the promoter region, which was found in 8/71 strains (one from serogroup A, three from lineage 3, and four from those classified as others), did not show any correlation with the expression of the protein.

21/40 convalescent sera recognized the recombinant protein in Western blot, while only 2/10 of sera from healthy people recognized it. Thus, GNA1870 may be immunogenic in patients recovering from meningococcal disease.

The Protein Induces Bactericidal Antibodies and Confers Passive Protection in the Infant Rat. The antisera obtained immunizing with the recombinant protein were tested for their ability to induce complement-mediated killing of encapsulated *N. meningitidis* strains. Representative strains from the high, intermediate and low expressors were selected for the assay using Western blotting. The differential expression of the protein on the surface of the selected strains was confirmed by FACS® analysis (Fig. 4). As shown in the figure, MC58, a representative of the high expressor strains was killed with high efficiency by the serum diluted up to 1/64,000, NZ394/98, a representative of the intermediate expressors was also killed with high efficiency, by the serum diluted up to 1/16,000 and even strain 67/00, a representative of the low expressor strains was killed by the antiserum diluted up to 1/2,048. A control strain, where the *gna1870* gene had been knocked out, was not killed by the same antiserum. To determine whether the sera were also able to confer protection in vivo, we tested them for their ability to induce passive protection in the infant rat model using two serogroup B and one serogroup C strains. Protection was achieved with all strains and the results obtained with one of the strains are shown in Table II. No bacterial colonies were recovered from the blood of the rats passively immunized with the anti GNA1870, while most of the controls were bacteremic. Similar results were obtained with two other strains (data not shown).

Table I. *Level of Expression of GNA1870*

Strains	High	Intermediate	Low
ET-5	9/9	0/9	0/9
Lineage 3	7/9	1/9	1/9
ET-37	2/3	1/3	0/3
A4	0/4	2/4	2/4
Other	6/15	7/15	2/15
N. gonorrhoeae	0/1	0/1	1/1
N. cinerea	0/1	0/1	1/1
N. lactamica	1/1	0/1	0/1
Total	25/43 (58%)	11/43 (25.5%)	7/43 (16.5%)

Gene Distribution and Protein Diversity. *gna1870* gene was detected by PCR in 71/71 *Neisseria* strains. The strain panel mostly includes serogroup B strains, a few strains of serogroups A, C, Y, W-135, and Z, and one strain each of *N. gonorrhoeae* and *N. cinerea*. In *N. lactamica* we could detect a band by Western blotting, but we failed to amplify the gene. Nucleotide sequence of the gene was determined in all 71 strains. Computer analysis of the *gna1870* encoded peptides, using Kimura and Jukes-Cantor algorithm divided the family in three variants (Fig. 5). Strains MC58, 961–5945, and M1239 were arbitrarily selected as type

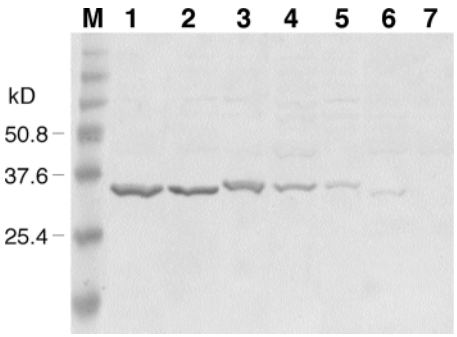

Figure 3. Western blots of a gradient SDS-PAGE gel loaded with total cell lysates of strains expressing high (lanes 1 and 2), intermediate (lanes 3 and 4), and low (lanes 5 and 6) levels of GNA1870. Lane 7 contains a total cell lysate from strain MC58 in which the gene encoding GNA1870 has been inactivated. Lane 1 strain MC58; lane 2 strain H44/76; lane 3 strain NZ394/98; lane 4 strain 961–5945, lane 5 strain 67/00, lane 6 strain M1239, lane 7 MC58*Δgna1870*.

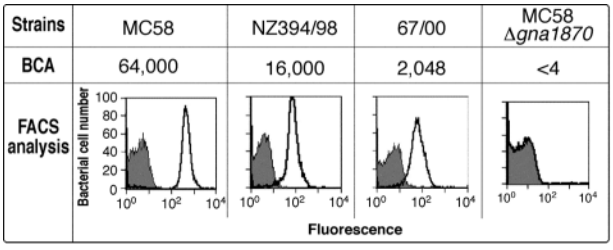

Figure 4. Bactericidal activity and FACS® analysis results of three strains expressing high, intermediate or low levels of GNA1870, based on Western blot of whole-cell lysates with antiserum against GNA1870. Shaded profiles are the negative controls obtained by reaction with the preimmune sera; nonshaded profiles show binding of immune sera. The intermediate expressor NZ394/98 and the low expressor 67/00 have identical amino acid sequences, which are 91.6% homologous to MC58.

Table II. *Passive Protection of Infant Rats Challenged with 5 × 10^7 CFU of Serogroup C Strain 4243*

Pretreatment	Dose of Ab (100 μl per rat) or serum dilution	No. bacteremia/total	CFU/ml (geometric mean)
PBS	–	5/5	450,000
Neg. control serum	1:10	5/5	500,000
Anti-PorA (mAb (P1.2)	2.0 μg	1/5	3
	1:10	0/5	<1
Anti-GNA1870	1:50	0/4	<1

Blood cultures were obtained 18 h after challenge.

strains for variants 1, 2, and 3, respectively. The sequence diversity between the three type strains is shown in Fig. 6. Amino acid identity was 74.1% between variant 1 and variant 2, 62.8% between variant 1 and variant 3, and 84.7% between variant 2 and variant 3. Sequences within each variant were well-conserved, the most distant showing 91.6, 93.4, and 93.2% identity to their type strains, respectively. *N. cinerea* belongs to variant 1, and shares 96.7% homology with MC58. As shown in Fig. 5, variant 1 harbors all strains from hypervirulent lineages ET-5, most lineage 3 strains, the serogroup A strains, two recent isolates of W-135 and one ET-37 strain. Variant 2 harbors all strains from the hypervirulent complex A4, from serogroups Y and Z, one old W-135 isolate and four ET-37 strains. Variant 3 harbors four unique ST strains, one ET-37 strain, one lineage 3 strain, and gonococcus.

Bactericidal Activity is Variant-specific. One sequence of each variant was expressed in *E. coli* as His-tag and used to immunize mice. The resulting antisera were used to test immunological cross-reactivity between strains of the three variants by FACS® and bactericidal assay. As shown in Fig. 7, by FACS® analysis, all strains were recognized by each antiserum, however, the degree of recognition varied considerably, usually reflecting the amino acid homology between the proteins. In fact, the antiserum against the variant 1 protein (Fig. 7, first row) recognized very well the MC58 strain, to a lower extent the 961–5945 strain, which is 74.1% homologous and, to a lesser extent, the strain M1239 that has an amino acid identity of 62.8%. A similar trend was found for antisera against variants 2 and 3 (rows 2 and 3, respectively, of Fig. 7), although with the anti variant 2 serum the differences were not striking. A monoclonal antibody against the capsule recognized equally well the three strains (Fig. 7, row 4), while a serum against the cytoplasmic protein NMB1380 used as negative control did not recognize them (Fig. 7, row 5). The *gna*1870 knockout mutants were not recognized at all by their specific sera (Fig. 7, row 6).

The differences in immuno-recognition between the three variants were more evident by bactericidal assay. As

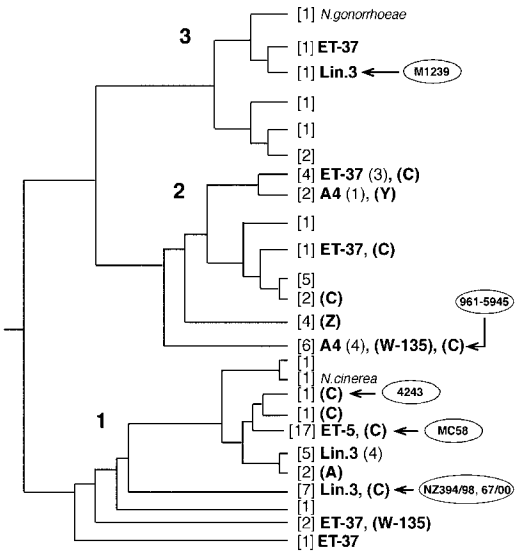

Figure 5. Dendrogram showing the strain clustering according to GNA1870 protein distances. 1, 2, and 3 indicate the three variants. Numbers in square brackets indicate the strains with identical sequence present in each branch of the dendrogram. Hypervirulent lineages are indicated, followed by the number of strains when this is different from the total number. Serogroups other than B are also shown. The three type strains (MC58, 961–5965, and M1239) and other strains used in the reported serological analysis and infant rat studies are within circles.

shown in Table III, the serum against each variant was able to induce efficient complement-mediated killing of the homologous strain (titers ranging between 16,000 and 64,000), while the activity was low (titers of 128–2,048) or absent (titers <4) against strains of the other variants. Moreover, in the case of variant 1, we also showed that the most distantly related strains, located at the bottom of the dendogram in Fig. 5, were killed by the anti-MC58 antiserum (titers of above 2.048).

As predicted from the close amino acid homology, the cross-bactericidal titers between variants 2 and 3 were higher than the others. When human complement was used, bactericidal titers of 4,096, 256 and 512 were obtained with variants 1, 2, and 3, respectively, using the homologous strains of Table III. No titers were detected against the heterologous strains.

Discussion

Vaccination against *N. meningitidis* serogroups A, C, W-135, and Y is likely to enter routine use once the conjugate vaccines against these strains will complete the ongoing clinical trials. This will leave disease caused by serogroup B meningococcus, which causes ~50% of invasive disease as the only one not preventable by vaccination. Given the chemical composition of the serogroup B capsule, which is identical to a polysaccharide present in human tissues, development of universal protein-based vaccines against serogroup B *N. meningitidis* are desirable and urgent.

Conventional approaches to protein-based vaccine development have delivered OMV vaccines which, although

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                                  +1
variant 1  -19:MNRTAFCCLSLTTALILTACS.....SGGGGVAADIGAGLADALTAPLDH: 26
variant 2  -19:MNRTAFCCLSLTAALILTACS.....SGGGGVAADIGAGLADALTAPLDH: 26
variant 3  -19:MNRTAFCCLSLTTALILTACSSGGGGSGGGGVAADIGTGLADALTAPLDH: 31

variant 1   27:KDKGLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGD...SLNTGKLKNDKV: 73
variant 2   27:KDKSLQSLTLDQSVRKNEKLKLAAQGAEKTYGNGD...SLNTGKLKNDKV: 73
variant 3   32:KDKGLKSLTLEDSIPQNGTLTLSAQGAEKTFKAGDKDNSLNTGKLKNDKI: 81

variant 1   74:SRFDFIRQIEVDGQLITLESGEFQVYKQSHSALTAFQTEQIQDSEHSGKM:123
variant 2   74:SRFDFIRQIEVDGQLITLESGEFQIYKQDHSAVVALQIEKINNPDKIDSL:123
variant 3   82:SRFDFVQKIEVDGQTITLASGEFQIYKQNHSAVVALQIEKINNPDKTDSL:131

variant 1  124:VAKRQFRIGDIAGEHTSFDKLPEGGRATYRGTAFGSDDAGGKLTYTIDFA:173
variant 2  124:INQRSFLVSGLGGEHTAFNQLP.DGKAEYHGKAFSSDDAGGKLTYTIDFA:172
variant 3  132:INQRSFLVSGLGGEHTAFNQLP.GGKAEYHGKAFSSDDPNGRLHYSIDFT:180

variant 1  174:AKQGNGKIEHLKSPELNVDLAAADIKPDGKRHAVISGSVLYNQAEKGSYS:223
variant 2  173:AKQGHGKIEHLKTPEQNVELAAAELKADEKSHAVILGDTRYGSEEKGTYH:222
variant 3  181:KKQGYGRIEHLKTLEQNVELAAAELKADEKSHAVILGDTRYGSEEKGTYH:230

variant 1  224:LGIFGGKAQEVAGSAEVKTVNGIRHIGLAAKQ:255
variant 2  223:LALFGDRAQEIAGSATVKIGEKVHEIGIAGKQ:254
variant 3  231:LALFGDRAQEIAGSATVKIGEKVHEIGIAGKQ:262
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Figure 6. Sequence alignment of variant 1 (strain MC58), variant 2 (strain 961–5945), and variant 3 (strain M1239). Amino acid numbers initiate from the Cysteine predicted to be the first amino acid of the mature protein. Gray and black backgrounds indicate conserved and identical residues, respectively.

efficacious against homologous strains, are unable to induce complement-mediated bactericidal antibodies against heterologous strains, especially in infants and toddlers. The determination of the genome sequence of group B strain MC58 made available the entire repertoire of gene products and allowed the discovery of several novel antigens, which may be used alone or in combination for the development of new vaccines. Some of these were briefly described in initial publications (25), while others have been described in more detail (26, 40). Here we report the discovery and characterization of a novel antigen, that is present on the surface of all strains of *N. meningitidis* tested and that is a highly promising candidate for the development of protein-based vaccines against meningococcus.

GNA1870 May Be Sufficient to Immunize against Most Strains of N. meningitidis. The observation that GNA1870 is expressed on the surface by all strains tested to date and is a good target for bactericidal antibodies makes this protein an excellent vaccine candidate. However, there are two potential problems that may limit the possibility of using the antigen. The first is that the expression varies from strain to strain; the second is that there is sequence variability with little or no cross-protection between the different variants. Here we have shown that even strains expressing

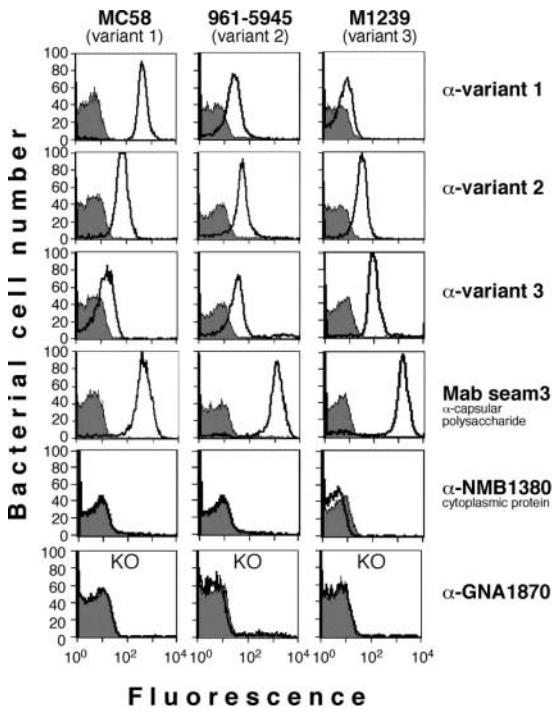

Figure 7. FACS® analysis of sera prepared against recombinant GNA1870 protein representative of variant 1 (first row), variant 2 (second row), and variant 3 (third row), using the type strain of variant 1 (MC58), variant 2 (961–5945), and variant 3 (M1239). Control sera against the capsular polysaccharide is shown in row 4 (monoclonal antibody Seam3). A negative control antiserum against a cytoplasmic protein is shown in row 5 (anti-NMB1380). Row 6 contains the knockout mutants (KO) of each type strain, probed with the homologous antiserum. Shaded profile are the negative controls obtained by reaction with the preimmune sera. Non-shaded profiles show reaction with immune sera.

Table III. *Cross Bactericidal Titers between GNA1879 Variants*

Sera	Bactericidal titers		
	MC58 (variant 1)	961-5945 (variant 2)	M1239 (variant 3)
Anti-variant 1	64,000	256	<4
Anti-variant 2	<4	16,000	128[a]
Anti-variant 3	<4	2,048	16,000

[a]The positive titer was found with sera from mice immunized with alum, but not in sera from mice immunized with Freund's adjuvant.

very low quantities of the protein are efficiently killed by the antisera, a result suggesting that even the low expressors have enough protein on the surface to be a target for complement-mediated attack. The sequence diversity, on the other hand, is limited to three variants, so that we believe it should be possible to induce immunity against all strains of meningococcus by immunizing with a combination of the three proteins. Although our focus is the development of a vaccine against meningococcus B, the data suggest that vaccination with three proteins would induce immunity against all serogroups of meningococcus (see Table II, infant rat protection using a C strain, 4243).

Features of the Gene and Protein. The presence of a putative Fur box in the promoter suggests that the expression of GNA1870 may be regulated by iron. However, preliminary experiments have shown that the expression of the protein does not increase in low iron conditions (data not shown). We have not been able to find a reason for the different expression of the protein by different strains. Analysis of the DNA sequences upstream from the gene did not show any feature that we could correlate with the differential expression.

An interesting feature of the protein is the presence of a stretch of four Glycines downstream from the lipidated Cysteine. Three or more consecutive glycines downstream from the lipidated Cysteine are present also in other five lipoproteins in *N. meningitidis*, namely the transferrin-binding protein B (TbpB), the outer membrane component of an ABC transporter NMB0623, the hypothetical protein NMB1047, the TbpB homologue GNA2132 (25), and the AspA lipoprotein (41). In none of these proteins is the poly-glycine stretch encoded by a poly-G tract, suggesting that this feature is not there to generate antigenic modulation. Proteins with this signature are widespread in prokaryotic organisms. A search performed on 22 complete bacterial genomes retrieved 29 lipoproteins with this motif in some but not all bacterial species. The organisms with these type of lipoproteins include both gram negative and gram positive bacteria such as *Haemophilus influenzae*, *Enterococcus fecalis*, *Mycobacterium tuberculosis*, *Listeria monocytogenes*, *Staphylococcus aureus*, while others such as *E. coli, Bacillus subtilis, Helicobacter pylori, Streptococcus pneumoniae, S. pyogenes*, and *Vibrio cholerae* have none. Most of the lipoproteins with this signature belong to ABC transporters, followed by proteins of unknown function. Although this common feature in the primary structure suggests a common role for the Glycine repeats, so far, the function is not known. However, it has been recently proposed that it may serve to guide the lipoproteins to a specific pathway of secretion and surface localization (42).

Conclusions. We have described a new protein of *N. meningitidis*, which is immunogenic during disease, is able to induce bactericidal antibodies in mice, and whose resulting antibodies confer passive protection against meningococcal bacteremia in infant rats. The protein is expressed by all strains tested. Overall these data suggest that protection against most *N. meningitidis* strains can be achieved by immunizing with three sequence variants of the protein. If human trials with this protein will induce an immune response similar to the one described here for mice, the development of protein-based vaccines against meningococcus may become a reality. In theory the three variants of GNA1870 could be enough to elicit immunity against all strains of meningococcus, however, a vaccine containing also other antigens may be more appropriate, because it would target more than one antigen per bacterium and therefore it would decrease the possibility of selecting escape mutants. A question that needs to be addressed in future studies is whether vaccines based on common proteins will immunize also against commensal *Neisseria* and whether this is desirable.

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