A Novel “Clip-and-link” Activity of Repeat in Toxin (RTX) Proteins from Gram-negative Pathogens

COVALENT PROTEIN CROSS-LINKING BY AN ASP-LYS ISOPEPTIDE BOND UPON CALCIUM-DEPENDENT PROCESSING AT AN ASP-PRO BOND

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Clinical isolates of Neisseria meningitidis produce a repeat in toxin (RTX) protein, FrpC, of unknown biological activity. Here we show that physiological concentrations of calcium ions induce a novel type of autocatalytic cleavage of the peptide bond between residues Asp414 and Pro415 of FrpC that is insensitive to inhibitors of serine, cysteine, aspartate, and metalloproteases. Moreover, as a result of processing, the newly generated amino-terminal fragment of FrpC can be covalently linked to another protein molecule by a novel type of Asp-Lys isopeptide bond that forms between the carboxyl group of its carboxyl-terminal Asp414 residue and the ε-amine group of an internal lysine of another FrpC molecule. Point substitutions of negatively charged residues possibly involved in calcium binding (D499K, D510A, D521K, and E532A) dramatically reduced the self-processing activity of FrpC. The segment necessary and sufficient for FrpC processing was localized by deletion mutagenesis within residues 400–657, and sequences homologous to this segment were identified in several other RTX proteins. The same type of calcium-dependent processing and cross-linking activity was observed also for the purified AxpIV A protein of Actinobacillus pleuropneumoniae. These results define a protein cleavage and cross-linking module of a new class of RTX proteins of Gram-negative pathogens of man, animals, and plants. In the calcium-rich environments colonized by these bacteria this novel activity is likely to be of biological importance.

Invasive infections by Neisseria meningitidis still belong to the most common causes of epidemic bacterial meningitis. Meningococci colonize the upper respiratory tract of about 10% of healthy asymptomatic carriers. Occasionally, however, the bacteria can penetrate across the mucosal barrier, invade the bloodstream, and pass the blood-brain barrier, causing life-threatening septicemia and/or meningitis. Many potential virulence factors involved in meningococcal pathogenesis have been studied in substantial detail over the past few decades. These comprise the polysaccharide capsule, adhesins like pili, OpA and OpC proteins, and the iron acquisition systems, respectively (1–5). In contrast to a number of Gram-negative bacterial pathogens, no proteinaceous exotoxins have yet been implicated in the pathogenesis of invasive meningococcal disease. In 1993, however, Thompson and colleagues (6, 7) discovered in meningococci two homologous secreted proteins, FrpC and FrpA (FrpC-like), that belong to the repeat in toxin (RTX) family of proteins characterized by variable numbers of carboxyl-proximal repetitions of a nonapeptide motif LXGXXG(D/N)DX. A number of RTX proteins have been shown to be involved in virulence of other Gram-negative genera, such as Actinobacillus, Bordetella, Escherichia, Moraxella, Morganella, Pasteurella, Proteus, and Vibrio (8, 9). In a previous study, we have shown that genes encoding FrpC-like proteins of variable size are present in most clinical isolates of N. meningitidis and that sera of a majority of patients who had invasive meningococcal disease contain antibodies specifically recognizing these proteins (10). Although the biological activity of FrpC-like proteins remains unknown, the findings suggested that it might be contributing to meningococcal carriage and/or disease.

We report here that the FrpC protein exhibits a novel type of “clip-and-link” self-processing activity. It is shown that FrpC undergoes a unique calcium-dependent autocatalytic processing at an Asp-Pro peptide bond that is accompanied by formation of high molecular weight oligomeric species of FrpC that contain subunits covalently cross-linked through a new type of isopeptide bond. The same type of calcium-dependent processing activity is demonstrated also for another RTX protein of unknown role in infections by Actinobacillus that also contains the novel protein cleavage and cross-linking module.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—The Escherichia coli K12 strain XL1-Blue (Stratagene), used throughout this work for DNA manipulation, was grown in Luria-Bertani (LB) medium supplemented with 150 μg of ampicillin/ml. Meningococci were grown on GCB medium (Difco) plates at 37 °C in an atmosphere containing 5% CO2. For analysis of FrpC processing in N. meningitidis, the wild type N. meningitidis strain MC58 and its previously described isogenic deletion variants ΔNMB0865 (lacking the frpC-like allele), ΔNMB1418 (lacking the frpC allele), and the double mutant lacking both frpC-like and frpC alleles were used (11).

The abbreviations used are: RTX, repeat in toxin; Frp, iron-regulated protein; CBD, chitin binding domain; Bis/Tris Propane, 1,3-bis(3-trishydroxymethyl)aminomethylpropane; CABS, 4-(cyclohexylamino)-1-butanesulfonic acid; MALDI, matrix-assisted laser desorption ionization; TOF, time-of-flight; FSD, postsource decay; TLCK, N-α-tosyl-l-lysine chloromethyl ketone hydrochloride; TPCK, t-1-tosylamide-2-phenylethyl chloromethyl ketone; E-64, N-(N-tosyl-l-2-carboxyoxirane-2-carbonyl)-l-leucylagmatine.
**Autocatalytic Cleavage and Cross-linking of FrpC**

**Plasmids—** The plasmid pTYB26-FLAG/FrpC was used for production of the FrpC protein tagged at its amino terminus with an artificial FLAG peptide (DYKDDDDK) inserted as a double-stranded synthetic oligonucleotide encoding the FLAG epitope (5'-TATGGTATATAAGATGACGAGAATACCGG-3' annealed with 5'-TACCGTGT- GATTGTCATCATCATCATATATGGA-3') into the NdeI site of pTYB26/FrpC (10). The pTYB2/FrpC-Apx construct was derived from pTYB26-FLAG/FrpC and was used for production of the truncated FrpCARTX protein lacking the 967 carboxyl-terminal residues of the RTX domain. FrpCARTX was tagged at the amino terminus by the FLAG peptide and at the carboxyl terminus by a His6 affinity purification tag. pFFAPxIVA1HisC was used for production of the recombinant carboxyl-terminally His-tailed ApxIVA protein in *E. coli* (12) and was generously provided by J. Frey from the University of Berne, Berne, Switzerland.

**Deletion and Site-directed Mutagenesis—** Deletions within the frpC alleles were obtained by PCR mutagenesis and/or by using the naturally occurring restriction sites in frpC to introduce in-frame deletions and insertions of suitable synthetic oligonucleotide adaptors. The constructions were called pTYB2/frpCΔα2–7Y where α and Y stand for the first and the last amino acid residues of the polypeptide segment that was deleted in the corresponding protein construct (e.g. the used X–Y deletions were 1–199, 200–397, 400–448, 659–862, 451–657, 541–562, and 564–657, respectively). Amino acid substitutions were introduced by site-directed PCR mutagenesis using the proofreading Vent DNA polymerase (New England Biolabs) and suitable pairs of mutagenic PCR primers. The PCR products with introduced substitutions were examined for the absence of other undesired mutations by DNA sequencing and introduced into the pTYB2/frpC-Apx constructs. Complete sequences and detailed schemes of the plasmid constructs will be provided upon request.

**Production and Purification of the FrpC-Derived Proteins—** The different FrpC-derived constructs were produced in the *E. coli* strain BL21 (DE3) transformed with the appropriate plasmid. Exponential 500-ml cultures grown at 30 °C in LB medium with 150 μg of ampicillin/ml were induced by isopropyl-β-D-thiogalactopyranoside (1 mM) for 4 h. The cells were washed twice in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 250 mM imidazole (pH 7.4) EDTA, resuspended in 50 mM Tris-HCl (pH 8.0), and loaded on a chitin bead column (New England BioLabs) and suitable pairs of mutagenic PCR primers. The PCR products with introduced substitutions were examined for the absence of other undesired mutations by DNA sequencing and introduced into the pTYB2/frpC-Apx constructs. Complete sequences and detailed schemes of the plasmid constructs will be provided upon request.

**Production and Purification of ApxIVA—** Recombinant ApxIVA was produced from pFFAPxIVA1HisC plasmid in isopropyl-β-D-thiogalactopyranoside-induced (1 mM) exponential cultures of *E. coli* BL21 (DE3) at 37 °C. Cells were washed twice in TN buffer with 5 mM EDTA (pH 7.4), resuspended in 50 mM Tris-HCl, 1 mM EDTA (pH 8.0), and disrupted by sonication. ApxIVA was extracted from insoluble cell debris by 8% urea in 50 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA (pH 8.0) and loaded on DEAE-Phenose. The urea concentration was decreased using a step gradient, and the column was washed with 50 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA (pH 8.0). The given FrpC-derived protein was then eluted by restoring 8 M urea in 50 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA (pH 8.0), and loaded on a chitin bead column. The extent of calcium-induced cleavage was calculated using the equation (U – PVU (%)) where U is the integrated signal intensity of the unprocessed FrpCARTX band in the negative control and PV is the integrated signal intensity of the unprocessed FrpCARTX band remaining in the processed protein sample. The integrated signal intensity of the gel background was subtracted from both values. The experiments were repeated four times, and the given values represent the average ± S.D.

**Mass Spectrometric Analysis—** Twenty-four individual protein bands per analyzed higher molecular weight protein species were excised from Coomassie R-250-stained SDS-polyacrylamide gels. The gel slices were pooled, chopped, and destained as described elsewhere (14). In-gel protein digestions were carried out with sequencing grade trypsin (Promega, 50 ng/μl) overnight at 37 °C. The generated peptides were extracted with 40% MeCN, 0.5% acetic acid; dried and reconstituted into 5% MeCN, 0.5% acetic acid prior to separation on a μRPC C2/C18 SC column (Shimadzu, Amersham Biosciences) in a linear gradient of MeCN in 0.5% acetic acid. The fractionated peptides were dried and reconstituted in 20 μl of 5% MeCN, 0.3% acetic acid prior to analysis by mass spectrometry.

**RESULTS**

**Calcium Ions Induce a Unique Processing of FrpC at an Asp-Pro Peptide Bond and Formation of Extremely Stable Higher Molecular Weight Forms of FrpC—** While purifying the

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Footnote: See [www.stanford.edu/~cuppiton/webmaxx8.htm](http://www.stanford.edu/~cuppiton/webmaxx8.htm).
recombinant FrpC protein, we were unable to separate the full-length FrpC (~198 kDa) from its ~150-kDa carboxy-terminal fragment formed during production in E. coli (10). It was, however, noted that the ~150-kDa fragment was more abundant when the chelating agent EDTA was omitted from purification buffers. As shown in Fig. 1, when the purified FrpC protein was incubated for 1 h with 2 mM Ca\(^{2+}\) ions instead of 1 mM EDTA, it was almost completely cleaved to the ~150-kDa carboxy-terminal and ~45-kDa amino-terminal fragments. More surprisingly, besides the FrpC fragments, several higher molecular weight forms of FrpC also formed during the calcium-induced processing (Fig. 1B, lanes 2, 4, and 6). These higher molecular weight forms were recognized both by the 9D4 anti-RTX monoclonal antibody recognizing the carboxy-terminal part of FrpC as well as by the anti-FLAG M2 antibody targeting an amino-terminal FLAG tag in the FrpC construct used. This suggested that upon calcium-induced cleavage, the FrpC protein could form oligomers that were not dissociated under the strongly denaturing conditions of sample preparation for SDS-PAGE. Furthermore neither the calcium-dependent FrpC cleavage activity nor its capacity to form higher molecular weight oligomers could be removed by various rather vigorous purification schemes. These comprised both native and denaturing conditions (e.g. 8 M urea solutions) and different combinations of up to three chromatographic steps, such as affinity purification of His\(_{6}\)-tagged FrpC on nickel-nitrotriacetic acid-agarose and/or chitin affinity purification of the intein-CBD-tagged FrpC, ion-exchange, and hydrophobic chromatographies, respectively (data not shown). This strongly suggested that the calcium-dependent cleavage and higher molecular weight formation activities were catalyzed by the FrpC protein itself and not by a contaminating E. coli component. The latter possibility was rather unlikely since no calcium-dependent protein processing activity has been reported for E. coli.

To characterize this activity of FrpC, the amino-terminal sequence was determined for the newly generated FrpC fragment resulting from calcium-induced processing and compared with the sequence of the co-purifying ~150-kDa FrpC fragment formed in E. coli that was incubated in 1 mM EDTA. Unexpectedly, while the co-purifying fragment exhibited amino-terminal sequence FAPWVKETK corresponding to cleavage between residues Cys\(^{305}\) and Phe\(^{306}\), a single and different amino-terminal sequence of PLALDLDGDG. This latter fragment could now be resolved by SDS-PAGE from the processed fragment (starting at Asp\(^{414}\), molecular mass = 151.2 kDa), and the two different fragments could not be resolved by SDS-PAGE.

To facilitate a more detailed analysis of processing and oligomerization of FrpC, a truncated FrpC RTX construct, lacking the entire RTX carboxy-terminal domain and consisting of only the first 862 residues of FrpC, was prepared. It was further fused to a self-excisable chitin affinity tag to allow purification on chitin beads. As documented in Fig. 2, again a carboxy-terminal fragment (~50 kDa) with the amino-terminal sequence FAPWVKETK was co-purified with FrpC (~95 kDa). As further shown in Fig. 2, in the presence of 2 mM Ca\(^{2+}\) the FrpC RTX protein was also fully competent for processing and oligomerization, yielding an expected ~45-kDa amino-terminal fragment, several higher molecular weight forms, and a newly generated carboxy-terminal fragment exhibiting the expected amino-terminal sequence of PLALDDGDG. This latter fragment could now be resolved by SDS-PAGE from the co-purifying fragment of FrpC RTX (starting at Phe\(^{306}\)) and the two fragments could be quantified separately. Moreover the size of the higher molecular mass products of FrpC RTX generated during processing also fell into a range allowing their better separation by SDS-PAGE (115–200 kDa). The FrpC RTX construct was, therefore, further used for detailed characterization of the calcium-induced processing of FrpC.

**Substitutions of the Asp\(^{414}\) and Pro\(^{415}\) Residues at the Processing Site Block the Calcium-induced Cleavage and Oligomerization of FrpC**—To analyze the sequence specificity of FrpC
processing, three individual substitutions disrupting the Asp-Pro cleavage site were constructed. Asp$^{414}$ was replaced by alanine (D414A) or glutamate (D414E) residues, and Pro$^{415}$ was substituted by alanine (P415A). As shown in Fig. 3, the substitutions of both Asp$^{414}$ and Pro$^{415}$ residues by an alanine abolished the calcium-dependent processing, while only a moderate reduction in the rate of FrpC$^\text{H9004 RTX}$ processing was observed upon substitution of Asp$^{414}$ by a Glu residue. Furthermore production of higher molecular weight forms could be observed also with the D414E variant. This shows that the processing activity of FrpC requires the presence of an acidic residue at position 414.

**Characteristics of the Calcium-dependent Processing Reaction**—To further analyze the calcium-dependent cleavage of FrpC, the kinetics, pH and temperature dependence, and the selectivity of the processing activity for divalent cations were determined. The processing products of FrpC$^\text{ΔRTX}$ formed under various conditions were separated by SDS-PAGE and quantified by densitometric analysis of Coomassie-stained gels. As shown in Fig. 4, addition of 2 mM free calcium ions resulted in rapid FrpC$^\text{ΔRTX}$ processing with a reaction half-time of about 5 min under the given conditions, and maximal conversion of about 85% was reached within 30 min. The temperature optimum of the processing reaction ranged between 35 °C and...
45 °C (Fig. 5A), and a rather broad optimal pH range between 5.5 and 8.5 was found (Fig. 5B). The calcium dependence of FrpC processing was further studied using a series of free calcium ion concentrations adjusted between 100 nM and 9.2 mM by a Ca²⁺-EDTA buffering system (WEBMAXC 2.10). As shown in Fig. 5C, cleavage of FrpC/RTX was induced by concentrations of free Ca²⁺ ions higher than 100 nM, and the processing rate continued to increase with rising Ca²⁺ concentrations up to the yield of about 80% FrpC/RTX conversion in 5 min at 9.2 mM Ca²⁺. As further shown in Fig. 5D, other divalent cations, such as Zn²⁺, Co²⁺, Mn²⁺, Ni²⁺, Mg²⁺, or Cu²⁺ of which some can be found as essential active site ions of metalloproteases, did not significantly influence the cleavage of FrpC. Among the seven tested divalent cations only cadmium could replace calcium and induced FrpC/RTX processing at about half the rate induced by equal Ca²⁺ concentrations (Fig. 5D).

Calcium-dependent Processing of FrpC Is Not Blocked by an Array of Prototype Protease Inhibitors—To get more information on the possible catalytic mechanism of FrpC processing, an array of typical inhibitors of all classes of conventional proteases was tested. However, as shown in Fig. 6, none of the inhibitors of serine proteases (e.g. antipain, aprotinin, 4-(2-aminoethyl)benzenesulfonyl fluoride, leupeptin, phenylmethanesulfonyl fluoride, N²-p-tosyl-L-lysine chloromethyl ketone (TLCK), and t-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)), of cysteine proteases (e.g. leupeptin, phenylmethanesulfonyl fluoride, TLCK, TPCK, and N-[(N-2-trans-carboxyoxirane-2-carbonyl)-L-leucyl]agmatine (E-64)), and aspartic proteases (pepstatin), respectively, could inhibit the cleavage of FrpC/RTX when used at the recommended or 10 times higher than usual concentrations (Fig. 6). The cleavage activity was neither inhibited by phosphoramidon, 1,10-phenanthroline, and 8-hydroxyquinoline-5-sulfonic acid, inhibitors of metalloproteases, nor was it blocked by bestatin, a typical inhibitor of aminopeptidases (Fig. 6).

The Processing Activity of FrpC Resides within the Segment Delimited by Residues 400–657—To identify the domain of FrpC accounting for the Asp 414-Pro 415 bond cleavage, a set of further truncated constructs was derived from FrpC/RTX (Fig. 7A) and examined for processing activity. As shown in Fig. 7B, neither large deletions within the 397 amino-terminal residues of FrpC nor removal of the 204 carboxyl-terminal residues of FrpC/RTX had any effect on the calcium-dependent processing activity. This was, however, ablated by deletion of the segment between residues 451 and 657 and/or by two shorter deletions within this segment comprising residues 451–562 and 564–657, respectively (Fig. 7). As expected, deletion of the processing site between residues 400 and 448 also abolished FrpC/RTX processing. The minimal FrpC segment accounting for the calcium-dependent processing of the Asp 414-Pro 415 bond was, thereby, delimited by residues 400–657.
Point Substitutions of Residues Potentially Involved in Binding of Calcium Ions Abolish FrpC Processing—Extensive screening of the self-processing segment of FrpC (residues 400–657) for the consensus sequence motifs of a wide range of known proteases and proteins undergoing autocatalytic cleavage failed to yield any useful hits (Network Protein Sequence Analysis, Institut de Biologie et Chimie des Protéines, Lyon, France). Since histidine residues are frequently involved in catalysis of peptide bond cleavage by other proteases, we examined whether any of the five histidine residues comprised within the processing segment of FrpC could play a role in its activity. However, individual substitutions of these histidine residues had no (H411L, H588A, and H651L) or very minor effect (H440V and H490L) on the rate of the calcium-induced processing as documented in Fig. 8.

In contrast to the missing protease signature motifs, however, the sequence comparisons of the processing segment revealed the presence of five potential calcium binding sequences, which exhibited various degrees of homology to the well defined EF-hand motif (Table I). Of these sequences, two exhibited a particularly high homology (residues 499–511 and 521–533) and had conserved all the key residues involved in

Fig. 6. Protease inhibitors do not block the calcium-dependent processing. FrpCARTX was preincubated for 2 min with the indicated protease inhibitors before free calcium concentration was adjusted to 2 mM to induce processing in the presence of the inhibitor for 5 min. The cleavage of FrpCARTX was quantified by densitometric analysis of SDS-polyacrylamide gels, and average values ± S.D. from four separate experiments are given. The concentrations of inhibitors were as follows: antipain, 100 μM; aprotinin, 10 μM; 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), 4 mM; leupeptin, 10 μM; phenylmethylsulfonyl fluoride (PMSF), 1 mM; TLCK, 500 μM; TPCK, 500 μM; E-64, 100 μM; pepstatin, 10 μM; bestatin, 130 μM; phosphoramidon, 1 mM; EDTA, 5 mM; EGTA, 5 mM; 1,10-phenanthroline, 5 mM; 8-hydroxyquinoline-5-sulfonic acid (HQSA), 3 mM.

Fig. 7. The calcium-dependent processing activity of FrpC is located within residues 400–657. A, schematic representation of the truncated FrpCARTX protein variants with deletions of residues 1–199, 200–397, 400–448, 451–657, 564–657, or 659–862, respectively. The deleted portions are indicated by the dashed lines separating the open bars. B, the truncated proteins were purified by affinity chromatography on chitin beads and incubated for 30 min at 37 °C in the absence (+) or in the presence (+) of 2 mM calcium ions. The protein samples were separated by SDS-PAGE (7.5%) and stained with Coomassie Blue. WT, wild type.
calcium ion coordination to the EF-hand consensus sites. Four of these residues were, therefore, individually replaced. As further shown in Fig. 8, single substitution of the Asp<sup>399</sup> residue by the oppositely charged lysine residue (D499K) and/or substitutions of the Asp<sup>510</sup> and Glu<sup>532</sup> residues by the neutral alanine residues (D510A and E532A) significantly reduced the rate of FrpC<sub>RTX</sub> processing. The substitution of Asp<sup>521</sup> by a lysine residue (D521K) or deletion of the Asp<sup>510</sup> residue (∆D510) reduced the processing capacity of FrpC<sub>RTX</sub> by about 3 orders of magnitude as determined by quantification of FrpC<sub>RTX</sub>D521K and FrpC<sub>RTX</sub>D510 processing after 600 min of incubation (data not shown). Complete loss of the activity then resulted from the deletion of Ala<sup>511</sup> in combination with a substitution of Asp<sup>462</sup> by a lysine residue within yet another predicted EF-hand (D462K + ∆A511). These results demonstrate that residues Asp<sup>399</sup>, Asp<sup>462</sup>, Asp<sup>499</sup>, Asp<sup>510</sup>, and Asp<sup>521</sup> play a crucial role in the processing activity of the protein most likely by being involved in binding the activating calcium ion(s).

**TABLE 1**

Potential EF-hand-like calcium binding sequences between residues 400 and 657 of FrpC

| Sequence<sup>a</sup> | Position<sup>b</sup> | Similarity<sup>c</sup> |
|---------------------|-------------------|-----------------|
| DHRNRGDI/TATgW      | 439–451           | 72              |
| DLNNGHIDN<sup>c</sup>gae | 462–474           | 74              |
| DSNGDNI<sub>NA</sub>NADa | 499–511           | 91              |
| DLNQDGIQANEL       | 521–533           | 89              |
| DNIII<sub>FR</sub>F<sub>K</sub>KEVEL | 585–597         | 75              |

<sup>a</sup> Putative calcium binding sequences of FrpC with different degrees of homology to the EF-hand consensus motif. Amino acid residues allowed in the EF-hand consensus sequence are given in uppercase letters, and the residues “not allowed” are in lowercase letters. Residues at positions 1, 3, 5, 7, 9, and 12, which are typically involved in coordination of calcium ions within well characterized EF-hand binding sites, are printed in bold.

<sup>b</sup> Positions of putative calcium binding sequences correspond to amino acid residues of FrpC from the FAM20 strain (6).

<sup>c</sup> Percentages of similarity of putative calcium binding sequences of FrpC to the EF-hand consensus sequence.

**FIG. 8.** Substitutions of residues predicted to bind calcium ions importantly affect the calcium-dependent processing. FrpC<sub>RTX</sub> variants bearing the indicated substitutions and/or deletions were purified by affinity chromatography on chitin beads and incubated at 37 °C for 30 min in the absence (−) or in the presence (+) of 2 mM free calcium ions. The protein samples were separated by SDS-PAGE (7.5%) and stained with Coomassie Blue. WT, wild type.

**Higher Molecular Weight Forms Consist of FrpC Subunits Covalently Cross-linked by a Novel Type of Asp-Lys Isopeptide Bond**—The unusual higher molecular weight oligomers of FrpC that formed during the calcium-induced processing reaction were extremely stable and could not be dissociated by harsh denaturing treatment, such as incubation for 15 min at 100 °C in the presence of 8 mM urea, 2% SDS, 100 mM dithiothreitol and subsequent separation by SDS-PAGE in gels containing 8% urea (data not shown). This suggested that at the course of the processing reaction the FrpC subunits were covalently linked. Therefore, the higher molecular weight forms generated by FrpC<sub>RTX</sub> were examined by mass spectrometry for the presence of peptide cross-links. The two most abundant higher molecular weight forms (indicated in Fig. 2 as Band 1 and Band 2, respectively) were separated by SDS-PAGE and proteolysed to fragments by trypsin. The obtained peptide mixtures were fractionated by reverse phase chromatography and analyzed by MALDI-TOF mass spectrometry. As illustrated in Fig. 9 by two examples of the numerous acquired spectra, the digests of one of the higher molecular weight forms (Band 1) contained an unpredicted tryptic fragment detected as a protonated species at m/z 1563.7 (Fig. 9A). This was absent in the spectra of tryptic digests of monomeric (unprocessed) FrpC<sub>RTX</sub>, and its mass did not correspond to any peptide predicted to form upon complete and/or partial digestion of FrpC<sub>RTX</sub>. Similarly the trypsin digests of the other excised higher molecular weight form of FrpC<sub>RTX</sub> (Band 2) contained another unpredicted ion species detected at m/z 1311.5 (Fig. 9B). Covalent structures of these suspect peptidic species could be derived from inspection of their respective daughter ion spectra obtained by PSD fragmentation and from examination of the masses of the respective immonium ions (Fig. 9). The identity and structure of the unpredicted tryptic fragments was further corroborated by analysis of PSD spectra of their ethylester derivatives, which confirmed the expected number of free carboxyl groups and fully supported the structures given in Fig. 9. These analyses revealed that upon calcium-induced processing at the Asp<sup>414</sup>-Pro<sup>415</sup> bond, the newly released carboxyl-terminal Asp<sup>414</sup> residue of the amino-terminal FrpC<sub>RTX</sub> fragment can form a novel isopeptide bond with free ε-amino groups of internal lysine residues of another FrpC molecule (or its fragment), yielding formation of the cross-linked higher molecular weight protein forms. Two different lysine residues, Lys<sup>663</sup> and Lys<sup>703</sup> (numbering refers to full-length FrpC sequence), were found engaged in isopeptide bond links to the carboxyl-terminal Asp<sup>414</sup> residue of the amino-terminal fragment of FrpC<sub>RTX</sub>. Moreover, when the cross-links formed by the further truncated construct FrpC<sub>Δ1–299Δ659−1829</sub> (consisting of residues 300–658) were analyzed, another two lysine residues, Lys<sup>256</sup> and Lys<sup>405</sup>, respectively, were found engaged in the isopeptide bond with Asp<sup>414</sup>. This suggests a promiscuity of the Asp-Lys isopeptide bond formation in that several different lysine residues of FrpC may be involved in formation of the cross-links within the higher molecular weight FrpC oligomers.

**The FrpC and FrpC-like Proteins Secreted by Meningococci Are Processed and Cross-linked in the Presence of Calcium Ions**—It was important to verify whether processing of FrpC and of its FrpC-like variant (initially called FrpA) and formation of their higher molecular weight forms also occurred during secretion by meningococci. This was indeed the case as documented in Fig. 10. Full-length forms of the FrpC

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and/or FrpC-like proteins were strongly detectable only in the culture supernatants of meningococci grown in RPMI 1640 medium supplemented with 0.4 mM EGTA that chelated free calcium ions, while the processed forms of the FrpC and FrpC-like proteins and their respective higher molecular weight forms were largely predominant in supernatants of cultures without added EGTA in which the secreted FrpC was exposed to calcium present in the phosphate-buffered

**Fig. 9.** The FrpC higher molecular weight forms contain subunits covalently cross-linked by a novel type of Asp-Lys isopeptide bonds. A, structure of the covalent Asp<sup>414</sup>-Lys<sup>703</sup> isopeptide bond cross-link detected within one of the higher molecular weight species (see Band 1 in Fig. 2) formed by FrpC<sub>ARTX</sub> in the presence of calcium ions. The tryptic fragment not matching any of the masses in the theoretical FrpC digest was detected in one of the peptide fractions at m/z 1563.7, and its structure was determined from the PSD daughter ion spectra. The immonium ions of the corresponding amino acid residues are indicated for Lys<sub>m/z</sub> 101) and Arg<sub>m/z</sub> 129), respectively. B, structure of a different Asp<sup>414</sup>-Lys<sup>663</sup> isopeptide cross-link within another higher molecular weight species of FrpC<sub>ARTX</sub> (Band 2, Fig. 2) that was determined by analysis of daughter ion PSD spectra of an unpredicted tryptic fragment detected at m/z 1311.5. The immonium ions for amino acid residues detected in PSD spectra of m/z 1311.5 are as follows: Val<sub>m/z</sub> 72), Asp<sub>m/z</sub> 88), Lys<sub>m/z</sub> 101), His<sub>m/z</sub> 110), Tyr<sub>m/z</sub> 136), and Arg<sub>m/z</sub> 129). Masses of fragments that lost the methanesulfenic acid (-64 Da) from oxidized methionine are given in parentheses (21). The residue numbering corresponds that of the full-length sequence of the FrpC variant from N. meningitidis FAM20 (GenBank™ accession number 1706913). Intens., intensity.
The role of the calcium-dependent cleavage and cross-linking step in the unknown biological function of these RTX proteins remains to be determined. Many proteins are, however, initially synthesized as inactive precursors that are activated by cleavage at one or several peptide bonds. Processing of proteins was shown to occur at bonds formed by various pairs of amino acid residues. However, no endoprotease cleaving an Asp-Pro bond has, to our knowledge, been described so far. It has been known for some time that the peptide bond between Asp and Pro residues is unstable in acidic solutions. Prolonged incubation (24 h) in 70% formic acid is indeed a method of chemical fragmentation of proteins at these bonds (15–17). The here-described calcium-dependent processing of the Asp414-Pro415 bond of FrpC appears, however, to be catalyzed by a novel proteolytic module present in a particular class of RTX proteins. The processing occurs at physiological pH and with reaction rates of about 2 orders of magnitude higher than the uncatalyzed chemical cleavage of the Asp-Pro bond.

Specific processing at the Asp-Pro bond was up to now reported only for two eukaryotic proteins, the heavy chain precursor of pre-α-inhibitor (pro-H3) and the MUC2 mucin. At this stage, it is difficult to decide whether the self-processing at the Asp-Pro bond in pro-H3, MUC2, and FrpC follows the same catalytic mechanism. The primary structures of pro-H3, MUC2, and FrpC are very different even for the portions surrounding the Asp-Pro cleavage site. Moreover pro-H3 and MUC2 do not require calcium ions for processing, and substitution of the Asp residue of the Asp-Pro bond of pro-H3 by a Glu residue completely abolished its processing (18, 19), while the same substitution only decreased the rate of FrpC cleavage. Furthermore the pH optimum of the processing reaction was between 3.5 and 4.5 for pro-H3, while the processing of FrpC occurred efficiently within a rather broad physiological pH range from 5.5 to 8.5. On the other hand, both low pH and calcium binding may serve the same purpose of modulating the specific structures of the two proteins to allow processing in a similar way.

The mechanism proposed for the chemical cleavage of the Asp-Pro bond in acidic conditions postulates that the reaction is initiated by a nucleophilic attack of the β-carboxyl group of the aspartate on the carbonyl carbon engaged in the imide bond with the proline residue. This is expected to yield an unstable intermediate and disruption of the peptide bond (20). Such cleavage reaction occurs exclusively at the peptide bond be-
between Asp and Pro residues because of the more basic character and the higher degree of imide nitrogen protonation of the proline residue in acidic environments as compared with any other amino acid residue (20). The cleavage has been proposed to yield formation of a reactive anhydride at the newly released carboxyl-terminal Asp residue of the fragment that is rapidly hydrolyzed to yield a carboxyl-terminal Asp residue.

In the case of pro-H3 processing, the formation of an anhydride at the carboxyl-terminal Asp has also been suggested and proposed to account for the formation of a covalent bond linking H3 to the chondroitin sulfate chain of the associated bikunin subunit of pre-α-inhibitor (19). It is plausible to expect that a reactive Asp anhydride also may be formed during the calcium-dependent autocatalytic cleavage of the Asp414-Pro415 bond in FrpC. This assumption would allow the proposal of a possible reaction scheme of processing and cross-linking of FrpC as outlined in Fig. 13. Binding of calcium to the processing module of FrpC would promote a conformational change in the protein that would allow interaction of an as yet uncharacterized residue(s) of FrpC with Pro415 and promote its protonation at physiological pH. This could promote a nucleophilic attack of the -carboxyl group of Asp 414 on the carbonyl carbon and rupture of the imide bond to Pro 415. A reactive anhydride at Asp414 could then form and either hydrolyze or get attacked by adjacent ε-amino groups of another FrpC molecule to form a new isopeptide Asp-Lys amide bond, such as those observed here to Lys663 and Lys703 residues of FrpC.

This model offers hypotheses that can now be tested experimentally. A question to answer is whether formation of the isopeptide bond between the processed amino-terminal fragment of FrpC and another FrpC molecule is also a catalyzed process or just a consequence of the high chemical reactivity of the putative anhydride at Asp414. An anhydride ring freely accessible to water molecules would be expected to hydrolyze before it could react with an amine group of another FrpC molecule. Moreover the isopeptide linking reaction of FrpC appears to be rather selective for free ε-amino groups of "itself" albeit accepting various FrpC lysine residues (e.g. Lys563 and Lys573 in FrpCΔRTX or Lys396 and Lys405 in FrpC1–299,659–1829, respectively). Indeed the high molecular weight forms of FrpC appear to be formed with comparable efficiency by purified FrpC and by crude FrpC in a cell lysate.

FIG. 11. Homologues of the FrpC clip-and-link activity module are also found in several other RTX proteins. Alignment of the FrpC segment accounting for the self-processing activity of FrpC (residues 400–657) with the homologous sequence segments of other RTX proteins from four other Gram-negative pathogenic species. The sequence homologues were identified in protein data bases by BLAST searches, and the sequences were aligned using the Lasergene software (DNASTAR) by the Clustal method. The conserved residues are printed in white within black squares. The numbering corresponds to the residue positions in the sequences of the aligned proteins. FrpC FAM20, FrpC of N. meningitidis strain FAM20, accession number 1706913; ApxIYA, ApxIYA of A. pleuropneumoniae 4074, accession number AAD01698; XfasA0970, XfasA0970 of X. fastidiosa Dixon, accession number ZP.00039036; RSc0249, RSc0249 of R. solanacearum GM1000, accession number NP.518370; RcaA, RcaA of R. leguminosarum 162Y10, accession number AAF36415 (all accession numbers are from GenBankTM).

FIG. 12. ApxIYA of A. pleuropneumoniae also undergoes the calcium-dependent cleavage at an Asp-Pro bond and formation of SDS-PAGE-resistant higher molecular weight oligomers. The recombinant ApxIYA protein was purified by a combination of ion-exchange and affinity chromatography and incubated for 2 h at 37 °C in the absence (−) or in the presence (+) of 2 mM calcium ions. The samples were separated by SDS-PAGE (7.5%) and stained with Coomassie Blue. HMW forms, high molecular weight forms of ApxIYA; ApxIYA, the entire 202-kDa ApxIYA; ApxIYA-Cter, carboxyl-terminal fragment of ApxIYA formed by the calcium-dependent cleavage between residues Asp638 and Pro639; ApxIYA-Nter, amino-terminal fragment of ApxIYA formed by the Asp638-Pro639 bond cleavage.
containing large amounts of unrelated proteins with free primary amine groups (data not shown) as well as upon secretion by meningococci into the RPMI 1640 culture medium containing rather high concentrations of free amino acids. This speaks in favor of a catalyzed formation of the isopeptide bonds between FrpC molecules. We have repeatedly found FrpC to migrate as a dimer under non-denaturing conditions of gel permeation chromatography and blue native electrophoresis (data not shown), and within FrpC dimers, some free ε-amino groups of lysine residues of the substrate FrpC molecule could be in the vicinity of the reactive anhydride as soon as it is generated upon rupture of the Asp-Pro bond. On the other hand, the observed efficacy of the amino-terminal fragment cross-linking to the FrpC molecules was rather low, and upon in vitro processing of the purified FrpC ΔRTX protein, most of the generated amino-terminal fragment was detected free.
1,10-phenanthroline and 8-hydroxyquinoline-5-sulfonic acid, which exhibit a high affinity toward Zn$^{2+}$ and a low affinity toward Cu$^{2+}$, were not able to inhibit the calcium-dependent processing of FrpC. Moreover removal of divalent cations from FrpC by the chelating agents followed by titration with calcium ions led to restoration of the processing activity of FrpC, while titration with zinc ions did not (data not shown), and processing of FrpC was not sensitive to phosphoramidon, which inhibits most microbial metalloproteases.

These results suggest that only calcium ions are required for FrpC processing at the Asp$^{511}$-Pro$^{512}$ bond. Binding of calcium ions is likely to promote a conformational change in the FrpC structure that allows the processing reaction to occur. Five potential EF-handlike calcium binding sequences were indeed identified within the FrpC self-processing segment. Two of these sequences that exhibit the highest homology to the consensus EF-hand motif, comprising residues 499–511 and 521–533, respectively, appear to play a crucial role in calcium-mediated activation of the autoprocessing capacity of FrpC. This is documented by the debilitating effects of the substitutions of residues Asp$^{499}$, Asp$^{510}$, Asp$^{521}$, and Glu$^{532}$ on FrpC processing. The arrangement of the potential EF-hand sites in FrpC appears, however, rather unusual. As outlined in the model proposed in Fig. 14, instead of being separated by a helix-loop-helix structure, which typically separates the calcium binding loops in EF-hand proteins, the two predicted calcium binding loops of FrpC could be separated by a very short segment of only 11 residues.

The present results suggest several directions in which the so far unknown biological function of the FrpC-like proteins could be searched. These proteins are secreted by meningococci during invasive disease and might be modulating the function of some host proteins by catalyzing the cross-linking of the host proteins or by covalently attaching their amino-terminal fragment to the host proteins. Alternatively the FrpC self-processing could serve just to form FrpC nets for some unknown purpose. Meningococci typically colonize the nasopharynx, can invade the bloodstream, and/or cross the blood-brain barrier. In all these niches free calcium ions are abundant. The calcium-dependent processing and cross-linking activity of FrpC may therefore be of importance for both the commensal and pathogenic lifestyles of meningococci.

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