The *Clostridium ramosum* IgA Proteinase Represents a Novel Type of Metalloendopeptidase*

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*Clostridium ramosum* is part of the normal flora in the human intestine. Some strains produce an IgA proteinase that specifically cleaves human IgA1 and the IgA2m(1) allotype. This prolylendopeptidase was purified from a broth culture supernatant, and N-terminal sequences of the native protein and tryptic fragments thereof were determined. A fragment of the *iga* gene encoding the IgA proteinase was isolated using degenerate primers in PCR, and the complete gene was obtained by inverse PCR. The identity of the *iga* gene was confirmed by heterologous expression in *Escherichia coli*. The deduced amino acid sequence indicated a signal peptide of 30 residues and a secreted proteinase of 133,828 Da. A typical Gram-positive cell wall anchor motif was identified in the C terminus. The presence of a putative zinc-binding motif His-Glu-Phe-Gly-His together with inhibition studies indicate that the proteinase belongs to the zinc-dependent metalloproteinases. However, the sequence of the *C. ramosum* IgA proteinase shows no overall similarity to other proteins except for significant identity around the zinc-binding motif with family M6 of metalloendopeptidases, and the unique sequence of the IgA proteinase in this area presumably establishes a new subfamily. The GC percentage of the *iga* gene is significantly higher than that for the entire genome of *C. ramosum*, suggesting that the gene was acquired recently in evolution.

IgA is the major class of immunoglobulin in human mucosal secretions. Two subclasses, IgA1 and IgA2, exist, and IgA2 is found in three allelic forms, A2m(1), A2m(2), and A2m(3), among which IgA2m(1) is expressed mainly in Caucasians (1). The IgA1 subclass is predominant in the upper respiratory tract and in serum, whereas more even proportions of IgA1 and IgA2 occur in intestinal and urogenital secretions (2, 3). A number of bacterial species that colonize mucosal membranes of man produce IgA1 proteinase. This group of postproline endopeptidases cleaves one of several Pro-Ser or Pro-Thr peptide bonds in the hinge region of human IgA1, including its secretory form, S-IgA1, within an inserted stretch of 13 amino acid residues lacking in IgA2. IgA1 proteinase-producing species include the three leading causes of bacterial meningitis, *Neisseria meningitidis*, *Hemophilus influenzae*, and *Streptococcus pneumoniae*; three commensal streptococci, *Streptococcus mitis*, *Streptococcus oralis*, and *Streptococcus sanguis*; *Gemella hemolysans*; and several species of *Capnocytophaga* and *Prevotella*. In addition, the urogenital pathogens *Neisseria gonorrhoeae* and *Ureaplasma urealyticum* produce IgA1 proteinase (reviewed in Refs. 4 and 5).

It is conceivable that IgA1 proteinases enable the bacterial species to escape specific immune defense on mucosal surfaces, although lack of relevant animal models has precluded definitive identification of their exact biological significance (5). Notably, human IgA1 cleaving activity among these bacteria has evolved in at least three independent evolutionary lineages, emphasizing the biological importance of these enzymes. Inhibition studies and molecular characterizations have shown that the *Hemophilus* and *Neisseria* IgA1 proteinases are homologous serine-type proteinases, the streptococcal IgA1 proteinases are mutually related metalloendopeptidases, and the enzyme from *Prevotella melaninogenica* (formerly *Bacteroides melaninogenicus*) is a cysteine proteinase (reviewed in Ref. 5).

*Clostridium ramosum* is a strict anaerobic, Gram-positive, spore-forming bacterium. It is part of the commensal flora in the human intestine (8, 9), and only rarely has it been associated with disease (10). Some strains of *C. ramosum* produce an IgA proteinase that cleaves human IgA1 and IgA2 allotype A2m(1) at a Pro-Val peptide bond at positions 221–222 just N-terminal to the hinge region (8, 11, 12). In IgA2m(2) and IgA2m(3), the Pro at position 221 is substituted by Arg, apparently rendering these allotypes resistant to cleavage by the IgA proteinase. Other *Clostridium* species may produce proteinase(s) with similar activity (13). The *C. ramosum* IgA proteinase is inhibited by high concentrations of EDTA, suggesting that it is a metalloproteinase (11). Otherwise, this enzyme has not yet been characterized.

Here we describe the purification, cloning, and characterization of the *C. ramosum* IgA proteinase. Analysis of the deduced translation product of the *iga* gene encoding the enzyme revealed that it is a metalloendopeptidase with a putative ex-
tended zinc-binding motif. The primary structure of the IgA protease shows no significant overall similarity to any other known metalloendopeptidase, including any IgA1 protease belonging to this class of proteolytic enzymes. Notably, however, the sequence of 30 residues around the zinc-binding motif shows up to 60% identity to the equivalent region of proteinases grouped into the family M6 of metalloendopeptidases, including PrtV proteinase of Vibrio cholerae and immune inhibitor A of Bacillus thuringiensis. The GC% of the iga gene is significantly higher than that reported for the C. ramosum genome, suggesting that the IgA protease gene was acquired recently in evolution through horizontal gene transfer.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains—**C. ramosum strain AK183, which produces an IgA protease that cleaves both human IgA1 and the IgA2m(1) allotype, was obtained from Dr. Y. Fujiyama (Kyoto, Japan). Bacteria were grown anaerobically at 37°C in 2× YT medium (14) supplemented with 0.05% sodium thiglycollate. The 2× YT medium was used because it is devoid of high molecular weight proteins that may complicate subsequent purification of the proteinase. Escherichia coli JM109 (Stratagene) was used as host for propagation of recombinant forms of plasmid pUC19. E. coli One Shot was used for cloning derivatives of the pCR-TOPO vector (Invitrogen, Groningen, The Netherlands), and E. coli BL21(DE3)pLYSs (R & D Systems Europe, Abingdon, UK) was used for the expression cloning. The E. coli strains were grown in 2× YT or LB medium (14) supplemented with antibiotics when appropriate.

**Preparation of the IgA Proteinase—**The cell-free supernatant of a 5-liter culture of C. ramosum was obtained by centrifugation (6,000 × g, 30 min, 4°C). Solid (NH₄)₂SO₄ was added stepwise to 47% saturation, in which a pilot experiment was found to precipitate the IgA proteinase, and NaN₃ was added to a final concentration of 0.5%. The precipitate formed overnight at 4°C was collected by centrifugation (6,000 × g, 45 min, 4°C), dissolved in 0.05 m Tris-HCl, pH 8.2 (buffer T), dialyzed against the same buffer, and stored at −20°C until used. The ammonium sulfate cut was further fractionated by size exclusion chromatography on a column (1.6 × 50 cm) of Sepharose 12 (prep grade; Amersham Biosciences) equilibrated in buffer T. Eluent fractions containing the IgA proteinase were identified by the capacity to cleave human IgA1 (see below). Fractions with maximal IgA1 cleaving activity were pooled and submitted to anion exchange chromatography on a Mono-Q column (Amersham Biosciences) equilibrated with buffer T. Protein fractions eluted with a gradient of NaCl (0–1 M) in buffer T were analyzed for IgA1 cleaving activity.

**IgA Proteinase Activity Assays—**IgA proteinase activity was detected by its capacity to cleave purified human myeloma IgA1. Briefly, IgA proteinase test samples were incubated with myeloma IgA1 at 0.5 mg ml⁻¹ in buffer T at 37°C overnight, and cleavage was subsequently detected by the presence of characteristic Fc and Fd fragments as revealed by SDS-PAGE (15). This assay was used to determine the susceptibility of the recombinant IgA proteinase to inhibition by human α₂-macroglobulin and various synthetic compounds including specific inhibitors of serine proteases (phenylmethylsulfonyl) fluoride, 3,4-di-chloroisocoumarin, and Pefabloc), cysteine peptidases (E-64 and iodoacetamide), and metallopeptidases (EDTA, 1,10-phenanthroline, phosphoramidon, 2-(N-hydroxy-carboxamido)-4-methyl pentanoyl-1 Ala-Gly-NH₂ (Zincov), N-hexozybenzocarbonyl-Pro-Leu-Glu-hydroxamate, and p-aminobenzoyl-Gly-Pro-n-Leu-u-Ala-hydroxamic). Pefabloc was purchased from Roche Molecular Biochemicals, Zincov from Merck, 1,7-phenanthrolin was from GFS Chemicals (Powell, OH), and the other compounds were from Sigma. The α₂-macroglobulin was titrated on trypsin prior to use and found to be 60% active, and incubation time before adding IgA1 substrate was up to 5 h.

For quantitative purposes, IgA proteinase activity was titrated using a previously described assay involving enzyme-linked immunosorbent assay technology (16). Briefly, serial dilutions of test sample were incubated with an equal volume of myeloma IgA1 substrate (50 μg ml⁻¹ in buffer II) at 37°C for 24 h with an amount of the partially purified recombinant proteinase capable of cleaving completely 0.5 mg of human IgA1 within 2 h. The proteins used were human IgG, IgD, IgE, IgM, α₂-macroglobulin, α₁-protease, α₁-antichymotrypsin (all from Athens Research and Technology, Athens, GA) and fibrinogen, bovine albumin, carboxymethylated lysozyme, collagen type I and IV, oxidized insulin a-chain, and gelatin from Sigma. After incubation, the reaction was stopped by boiling in reducing sample buffer, and the integrity of the proteins was analyzed by SDS-PAGE.

**Amino Acid Sequencing—**The most active fractions eluted from the Mono-Q column were subjected to reducing SDS-PAGE, blotted onto ProBlott membranes (PerkinElmer Life Sciences), and stained with Coomassie Blue. The band corresponding to the presumed IgA proteinase was excised using an ABI 477A/120A protein sequencer (PerkinElmer Life Sciences). For generation of tryptic peptides, the band in the polyacrylamide gel stained with Coomassie Blue was excised from several lanes and digested in situ as described (17). In brief, after washing with a mixture of ammonium bicarbonate and acetonitrile, pieces of the gel were shrunk with acetonitrile and dried completely. A solution containing modified trypsin (Promega, Madison, WI) was allowed to soak into the gel pieces. After incubation overnight at 37°C, generated peptides were recovered by extraction and separated by narrow bore RP-HPLC using the SMART system (Amersham Biosciences). Amino acid sequencing of selected peptides was performed as described above.

**Southern Blot Analysis—**Unless otherwise stated, the DNA manipulations were performed according to Sambrook et al. (14). Whole-cell DNA from C. ramosum was isolated, digested with EcoRI, and subjected to agarose gel electrophoresis and Southern blot analysis including hybridization at high and low stringency as described previously (18). As probes we used a 5.1-kb fragment of the S. sanguis strain ATCC 10556 iga gene (18) and a PCR product of genomic DNA from C. ramosum strain AK183 amplified with the primers 5'-AACGTGGTTCGCGAATTAAGTC-3' and 5'-AGCTTCCTTC-3', identified in the present study, using the Expand Long Template PCR System as recommended by the supplier (Roche Molecular Biochemicals). The DNA probes were purified after agarose gel electrophoresis (QIAEX II Gel Extraction Kit, Qiagen, Valencia, CA) and labeled with [³²P]dCTP (Random Labeling Kit, Roche Molecular Biochemicals).

**Sequencing the IgA Proteinase Gene—**Several degenerate primers were designed to reverse translation of the obtained amino acid sequences of the peptides analyzed were purchased from DNA Technology (Aarhus, Denmark). The primers were combined in pairs of a forward and a reverse one and used in PCRs containing 100 ng of genomic DNA and 30 pmol of each primer using Ready To Go PCR beads (Amersham Pharmacia Biotech) and subjected to the following cycling parameters for 30 cycles: 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, with an initial denaturation step at 94°C for 5 min and a final extension at 72°C for 8 min. The resulting PCR products were cloned into the E. coli plasmid vector pCR-TOPO using the TOPO-TA Cloning Kit (Invitrogen). For inverse PCR, the genomic DNA was digested with either MspI or EcoRI, and 50 ng of the resulting fragments was circularized in a 20-μl reaction volume (Rapid DNA Ligation Kit, Roche Molecular Biochemicals). The self-ligated mixture was purified with Wizard Minicolumns (Promega) and used as template in the inverse PCR with primers pointing outwards using either Ready To Go PCR beads or the Expand High Fidelity PCR System (Roche Molecular Biochemicals) and the same cycling parameters as described above except that the annealing temperature was 60°C. The inverse PCR products were cloned into E. coli plasmids pCR-TOPO or pUC19. The plasmid DNA was used for construction of plasmid clones with nested, unidirectional deletions for sequencing the insert of recombinant plasmids using the universal M13 primers.

Plasmid DNAs for sequencing were prepared as recommended by the supplier of the sequencing kit, and PCR products were purified with Wizard Minicolumns (Promega). Individual sequence reactions on plas-
mid DNA were performed with the Taq DyeDeoxy-Terminator cycle sequencing Kit (PerkinElmer Life Sciences), whereas Thermo Sequenase Dye Terminator Cycle Sequencing Kit (Amersham Biosciences) was used for sequencing PCR products. Sequence reactions were analyzed with an ABI PRISM 377 DNA sequencer (PerkinElmer Life Sciences). For sequencing primers, we used the universal M13 sequencing primers as well as oligonucleotides designed on the basis of preceding sequences. The DNA sequence was determined for both strands of the iga gene. Computer analysis of the sequences was performed with programs included in the GCG package (Genetics Computer Group, Madison, WI). BLAST and PSI-BLAST (available on the World Wide Web at www.ncbi.nlm.nih.gov/BLAST/) were used for data base searching.

Expression of the ORF—For expression in *E. coli* of the ORF, we used the vector pGEX-5T, which is designed to express a recombinant fusion protein consisting of a histidine hexapeptide and glutathione S-transferase followed by the amino acid sequence of interest (19). The primers were CGGTGATGAGACATTTGAGCCATCGCAAAACG-3’ and 5’-GCTTAAAGGCTTATCGGTCATTCCG-5’ were used in a PCR on genomic DNA from strain AK183 to amplify a fragment encoding the presumed secreted form of the IgA protease, and, in addition, the primers added a BamHI and an XhoI restriction site, respectively. For the PCR, we used the Pwo polymerase as recommended by the supplier (Roche Molecular Biochemicals). The vector and PCR product were digested with BamHI and XhoI, ligated, and transformed into *E. coli* JM109. Colonies harboring the correct recombinant plasmid, termed pGEX-5T-iga, were identified by restriction analysis of plasmid DNA. The plasmid DNA was subsequently used to transform *E. coli* BL21(DE3)pLysS, and expression of the recombinant protein was induced in a culture with an *A_{opt} of 0.6 by adding IPTG to a final concentration of 1 mM. After an additional 4 h of growth, the cells were pelleted, resuspended in phosphate-buffered saline, pH 7.3, and disrupted by mild sonication. The supernatant of the lysate was tested for IgA cleaving activity as described above.

The recombinant IgA protease was partially purified using affinity chromatography on glutathione-Sepharose (Amersham Biosciences). Briefly, the *E. coli* cells from 3 liters of culture grown for 4 h at 25 °C after induction of expression by IPTG were suspended in 50 ml of phosphate-buffered saline and disrupted using a French press. Cell debris was removed by ultracentrifugation (105,000 × g for 60 min), and recombinant IgA protease was separated using an on-column cleavage and purification procedure as recommended by the manufacturer. Thrombin was removed using benzamidine-Sepharose (Amersham Biosciences).

Site-Directed Mutagenesis—Three mutants of the *C. ramosum* IgA protease, H539A, D550A, and E551A, were generated. Mutator oligonucleotide primers were designed to introduce restriction enzyme sites to facilitate subsequent screening for mutated DNA products.

For the H539A mutation, the codon CAC for histidine was replaced by GCC, a codon for alanine. In addition, a silent mutation in codon Ala981 was introduced by changing GCA into GCC, creating an Eco52I restriction site. CGCGCG. Outward facing primers for the H539A mutation were 5’-AACCTGTGCTCAATCTGGCCGCAAAGTTT-3’ and 5’-TGCTCGGTCCTCGGTGATAA-3’. For the D550A mutation, the codon GAT for aspartic acid was replaced by GCT, a codon for alanine. In addition, a silent mutation in codon Gly461 was introduced by changing GGT into GGG, creating an HinI/HindIII restriction site, creating an Eco52I and SalI restriction site, generating pGEX-iga-H539A, pGEX-iga-D550A, and pGEX-iga-E551A, had single amino acid substitutions at amino acids His128 to Ala, Asp981 to Ala, and Glu992 to Ala, respectively. The plasmids were transformed into *E. coli* BL21(DE3)pLysS, and expression of recombinant proteins was induced as described above.

**RESULTS AND DISCUSSION**

**Purification, Characterization, and Amino Acid Sequence Analysis of the *C. ramosum* IgA Protease—** Differences in the proportion of secreted compared with cell-associated forms of the IgA1 protease produced have been observed for different species and strains of bacteria (20). Here we found that in the early stationary phase the majority of the IgA1 cleaving activity in *C. ramosum* strain AK183 was secreted into the medium (data not shown). The *C. ramosum* IgA protease was purified from culture supernatant by a combination of ammonium sulfate precipitation, size exclusion, and anion exchange chromatography. Eluent fractions containing the protease were identified by their ability to cleave human IgA1, releasing intact Fc and Fd fragments (analyzed by SDS-PAGE), and the activity in fractions was determined by titration of the ability to cleave human IgA1 (analyzed by the enzyme-linked immunosorbent assay-based assay). The titer of IgA protease activity in the initial 5 liters of culture supernatant was 8, and in the peak activity fraction (0.5 ml) upon anion exchange it was 128. This modest increase in activity suggested a loss of enzyme activity during the process of purification. Because *C. ramosum* is a strictly anaerobic bacterium, we speculated that the enzyme might regain its activity if subjected to reducing conditions. However, we found that preincubation with neither 1 mM β-mercaptoethanol nor 1 mM dithiothreitol had any influence on its capacity to cleave human IgA1. The loss of activity during purification remains unexplained.

The IgA protease was active at neutral pH, and it retained activity upon storage at −20 °C for several weeks. It has been previously shown that 100 mM EDTA inhibits the activity of the *C. ramosum* IgA protease (11). We found that the IgA1 cleaving activity was completely inhibited by 0.5 mM EDTA, suggesting that the enzyme is a metalloproteinase. More detailed enzymatic characterization of the IgA protease activity was performed using the partially purified recombinant form of the enzyme (see below).

Reducing SDS-PAGE analysis of the Mono-Q fractions revealed that the intensity of a band corresponding to a protein of 130 kDa correlated with the IgA1 proteinase activity, suggesting that this band represented the IgA protease (Fig. 1). We found that the IgA1 cleaving activity was completely inhibited by 0.5 mM EDTA, suggesting that the enzyme is a metalloproteinase. More detailed enzymatic characterization of the IgA protease activity was performed using the partially purified recombinant form of the enzyme (see below).

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EIPNTAGG, and EYTGAY were obtained for three of the tryptic peptides. None of the sequences shared significant similarity to other bacterial IgA1 proteinases or to any known proteins, as revealed by searching the GenBank™ data base.

The iga Gene Sequence from C. ramosum Strain AK183—Although the molecular mass and catalytic mechanism of the C. ramosum IgA proteinase was similar to that of the IgA1 proteinase from streptococcal species (18, 21–23), the C. ramosum iga gene encoding the IgA proteinase showed no homology to the streptococcal iga genes. Even when using hybridization at very low stringency conditions, genomic DNA from C. ramosum strain AK183 did not hybridize with the iga gene from S. sanguis in a Southern blot analysis (data not shown). To isolate the C. ramosum iga gene, the N-terminal amino acid sequences obtained for the putative IgA proteinase and the tryptic fragments of it were used to design degenerate primers for PCR amplification of a part of the C. ramosum iga gene using genomic DNA from strain AK183 as template. Forward primer 5'-ATGGGIGTITAYAAYAAY-3' was deduced from reverse translation of the amino acid sequence MGVYNN from the N-terminal sequence of the mature protein, and reverse primer 5'-RAARTGTRGAICCRTAYCTC-3' was deduced from the sequence EYGFHYYF for one of the tryptic peptides. A single amplicon of ~1.2 kb was produced. The nucleotide sequence of this fragment was determined and used for design of primers for inverse PCR to obtain the complete iga gene sequence. Combined, a sequence of 4242 nucleotides was determined. To correct for errors that may occur due to imperfect fidelity of the DNA polymerases in the PCRs and which would be carried over in the cloning procedures applied in the sequencing strategy (see “Experimental Procedures”), the sequence obtained was used to design primers for PCR amplification of overlapping fragments of the AK183 iga gene. Direct sequencing of the amplicons revealed a total of five errors in the initial sequence.

The sequence contained a large ORF with the potential of encoding a protein of 1,234 amino acids. The N-terminal sequence of the 130-kDa protein as well as the N-terminal sequences of the tryptic fragments of it were all identified within the primary structure deduced from the ORF (Fig. 2). The ORF was preceded by typical promoter elements (26, 27). Another possible ATG start codon, located 45 nucleotides upstream of the proposed ATG start codon, was also identified.

A Southern blot analysis of genomic DNA of C. ramosum AK183 restricted with PstI, which has no recognition sites in the iga gene sequence determined, and hybridized with a 4-kb fragment containing the ORF showed a single band of 14 kb (results not shown), suggesting that the iga gene is a single copy gene in C. ramosum strain AK183.

Interestingly, the GC percentage of the iga gene was 43 compared with an overall GC percentage of 26 in the C. ramosum genome (28). This difference strongly suggests that the IgA proteinase gene in C. ramosum was acquired recently in evolution through horizontal gene transfer from another bacterium with a higher GC percentage.

Expression of the IgA Proteinase in E. coli and Characterization of the Recombinant Protein—To verify that the ORF in fact represented the C. ramosum iga gene, we performed heterologous expression in E. coli. The sequence encoding the presumed mature IgA proteinase (positions 537–4151 in Fig. 2) was amplified by PCR and cloned into the E. coli expression vector pGEX-5T. This vector is designed to express a recombinant fusion protein consisting of a histidine hexapeptide and glutathione S-transferase followed by the amino acid sequence of interest. The plasmid construct, termed pGEX-5T-iga, was transformed into E. coli BL21(DE3)LyS. Intracellular expression of the fusion protein was induced by IPTG, and after incubation the cells were disrupted by sonication. The resulting lysate showed IgA proteinase activity (Fig. 3), demonstrating that the ORF sequenced was the iga gene. In addition, N-terminal sequencing of the Fc fragment generated by the recombinant fusion protein revealed the sequence VPSTP. This sequence is identical to that previously reported for Fc induced by the C. ramosum IgA proteinase (11), indicating that the specificity of the recombinant proteinase was identical to the native one.

Relatively high expression of the active recombinant form of the IgA proteinase enabled us to perform a more detailed characterization of the enzyme activity. First, we confirmed that the IgA proteinase is highly specific for human IgA, since none of the other human immunoglobulins, including IgG, IgD, IgE, and IgM, were susceptible to cleavage. In addition, none of the other proteins tested (fibrinogen, albumin, collagen type I and IV, and two serpins, α1-proteinase and α2-antichymotrypsin) were cleaved by the IgA proteinase even after incubation for 24 h at an enzyme concentration sufficient to cleave 0.5 mg of human IgA1 in 2 h (data not shown). Especially significant was the lack of an effect on serpins, since this group of proteins possess a surface-exposed loop (the reactive site loop), which is readily cleaved even by nontarget proteinases with a restricted specificity like periodontain (29) and collagenase (30). These data, together with the lack of activity against unstructured polypeptides such as gelatin, carboxymethylated lysozyme (Fig. 4), and oxidized insulin 6-chain, indicate an exquisite specificity of the C. ramosum IgA proteinase. Such a narrow specificity limited to the hinge region of the IgA molecule is also a common feature of other IgA1 proteinases (4, 5).

It has been assumed that the C. ramosum IgA proteinase is a metalloproteinase solely on the basis of its inhibition by EDTA (11). To extend enzyme characterization, we investigated the effect of a broad range of class-selective inhibitors of serine proteinases, cysteine proteinases, and metalloproteinases on the activity of the partially purified recombinant IgA proteinase. At 1 mM concentration, none of the compounds tested (fibrinogen, albumin, collagen type I and IV, and two serpins, α1-proteinase and α2-antichymotrypsin) were cleaved by the IgA proteinase even after incubation for 24 h at an enzyme concentration sufficient to cleave 0.5 mg of human IgA1 in 2 h (data not shown). Especially significant was the lack of an effect on serpins, since this group of proteins possess a surface-exposed loop (the reactive site loop), which is readily cleaved even by nontarget proteinases with a restricted specificity like periodontain (29) and collagenase (30). These data, together with the lack of activity against unstructured polypeptides such as gelatin, carboxymethylated lysozyme (Fig. 4), and oxidized insulin 6-chain, indicate an exquisite specificity of the C. ramosum IgA proteinase. Such a narrow specificity limited to the hinge region of the IgA molecule is also a common feature of other IgA1 proteinases (4, 5).

The Amino Acid Sequence of the C. ramosum IgA Proteinase

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FIG. 2. Nucleotide sequence of the iga gene from C. ramosum strain AK183. The deduced amino acid sequence of the large ORF is shown below the gene sequence. The proposed /H11002 and /H11002 promoter sequences and the Shine-Dalgarno sequence are underlined. The inverted repeat structure downstream of the ORF is shown by divergent arrows. The cleavage site for the N-terminal signal peptide is indicated by an arrow. The putative zinc-binding motif is shown by triangles below the sequence, and the obtained N-terminal amino acid sequences are underlined and shown in boldface type.
target surface proteins to the cell wall (32). The sequence SPQTG at positions 1196–1200 presumably constitutes the sortase recognition site. It was previously reported that in *Clostridium difficile* the sortase appears to recognize SPXTG or PPTXT instead of the conventional LPXTG motif (33). In the *C. ramosum* IgA proteinase, a smaller spacer, DSN, separated this motif from a transmembrane domain, IFLWALLFVSAGVGETGITYAT, followed by a positively charged tail, NKKKKEHAE, at the C terminus. These features are in agreement with other presumed substrates for sortase-like proteins (33). Provided that the anchor motif is functional, the sortase cleaves at the Thr-Glu peptide bond in the recognition site and covalently links the threonine, and thereby the N-terminal part of the protein, to peptidoglycan in the cell wall (32, 34). However, we found that the majority of IgA proteinase activity in *C. ramosum* AK183 was released into the medium. Release of surface proteins with a typical Gram-positive cell wall anchor motif has been reported for the α and β antigens present in the protein complex of *Streptococcus agalactiae* (35–37), and *Streptococcus mutans* sheds surface antigen P1 and secretes exo-β-d-fructosidase (38, 39). The release of anchored surface proteins may be brought about by turnover of the peptidoglycan layer or by proteolytic cleavage of the proteins next to the anchoring. Provided that the cell wall anchor sorting signal in the *C. ramosum* IgA proteinase is functional, the mechanism by which the proteinase is released from the cell wall remains to be elucidated.

A putative zinc-binding motif was identified at positions 539–543 followed by an aspartic acid residue seven positions downstream in the sequence HEXXXHXXXGXXD and resembling the extended zinc-binding site typical for the metzincin group of metallopeptidases, but as a significant difference in the IgA proteinase there are four instead of three residues between the second His and Gly (40, 41). In all members of this clan with the exception of leishmanolysins, the third zinc ligand is His or Asp, located invariably six residues downstream of the HEXXH motif (42). In case of *C. ramosum* IgA proteinase, there are seven residues separating the second (His) and the third (Asp) zinc ligand. Nevertheless, the sequence encompassing the zinc-binding motif is remarkably similar to that of the PrtV proteinase of *V. cholerae* and the immune inhibitor A of *B. thuringiensis* (Fig. 6), each of which is a proteolytic member of clan MA. This significant similarity includes the presence of the conserved Gly, which allows the formation of the β-turn necessary to bring the zinc ligands together in this group of metallopeptidases (43, 44). Therefore, it can be predicted that HisG539, HisG541, and AspG550 of the *C. ramosum* IgA proteinase polypeptide chain form the metal binding site, while GluG540 is the active site residue.

To experimentally verify the prediction that HisG539 and AspG550 constitute part of the zinc binding motif and are therefore indispensable for the enzyme activity, we constructed and expressed mutant forms of the IgA proteinase in which these residues were individually replaced by alanine. As expected, neither of these two mutants possessed IgA1 cleaving activity (Fig. 7, lanes 4 and 5). Notably, however, the E551A mutant was fully active (Fig. 7, lane 6). These data corroborate the alignment-based predictions of the zinc-binding and catalytic residues (Fig. 6) and indicate that the IgA proteinase of *C. ramosum* can be included into clan MA. The endopeptidases from clan MA are also known as metzincins, because there is a conserved Met in a turn that underlines the active site (41, 45). However, this Met is absent in the IgA proteinase, indicating the uniqueness of this enzyme, which most likely establishes a new subfamily of metallopeptidases in family M6 of clan MA.

Currently, the family M6 consists of only three members listed in the MEROPS Data Base (available on the World Wide...
therefore the fragments migrate slightly differently in the gel.

different preparation of human IgA1 with a distinct glycosylation, and mass standards are shown in incubated with human IgA1 and analyzed by SDS-PAGE. Molecular E551A (\(5T-iga-H539A\) (lane 3), pGEX-5T-iga-D650A (lane 5), pGEX-5T-iga-E551A (lane 6), or pGEX-5T-vector (lane 7) induced with IPTG were incubated with human IgA1 and analyzed by SDS-PAGE. Molecular mass standards are shown in lane 1, and IgA1 incubated with buffer is shown in lane 2. In this assay compared with Figs. 3 and 5 we used a different preparation of human IgA1 with a distinct glycosylation, and therefore the fragments migrate slightly differently in the gel. Below the SDS-PAGE gel, a Western blot analysis of appropriate lysates probed with anti-His\(_{6}\) antibodies demonstrates that the lack of IgA1 cleaving activity was not due to deficiency in expression of the mutated proteinase. The position of the recombinant proteinase is indicated by an arrow.

Web at www.merops.co.uk) exemplified by PrtV proteinase of \(V.\) cholerae and immune inhibitor A of \(V.\) cholerae. This metalloendopeptidase activity can, however, be inferred indirectly from the parent that within the metalloproteinase class, the specificity of enzyme capable of cleaving both IgA1 and IgA2m(1) molecules. This seems to be a major advantage for this bacterium, because in the gut environment, a natural habitat of \(C.\) ramosum, both isotypes of IgA occur in comparable amounts. It remains to be examined whether strains of \(C.\) ramosum producing this proteinase preferentially colonize subjects homozygous for the IgA2m(1) allelle. Besides, the expression of the recombinant IgA proteinase facilitates production of a large amount of the active, recombinant protein in a pure form for further studies of this intriguing molecule.

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FIG. 6. Conserved region in \(C.\) ramosum IgA proteinase. A comparison is shown of the region around the predicted active site and zinc-binding domains (indicated by A and Z, respectively) of \(C.\) ramosum IgA proteinase (IgAPrt), \(B.\) thuringiensis immune inhibitor A (IhA; accession number X55436), and \(V.\) cholerae PrtV (PrtV; accession number Y00557), with a hypothetical secreted proteinase of \(S.\) coelicolor (ScPprt; accession number CAB51001) and those of PrtV-related proteinases obtained from the conceptual translation of sequences retrieved from genome data bases (available on the World Wide Web at www.ncbi.nlm.nih.gov/Microb_blast/unfinishedgenome.html): BaPrt1 from \(B.\) anthracis, BaPrt2 from \(B.\) anthracis, BstePrt from \(B.\) stearothermophilus, ClaPrt from \(C.\) acetobutylicum, and SputPrt from \(S.\) putrefaciens. The sequences were aligned using the ClustalW multiple sequence alignment tool. The arrows above the sequences indicate Gly and Met residues conserved in the metallozin family of metalloepitido-
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*C. ramosum IgA Proteinase*