The Immunoglobulin D-binding Part of the Outer Membrane Protein MID from Moraxella catarrhalis Comprises 238 Amino Acids and a Tetrameric Structure*

Received for publication, April 22, 2002, and in revised form, July 2, 2002
Published, JBC Papers in Press, July 10, 2002, DOI 10.1074/jbc.M203558200

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Moraxella catarrhalis IgD-binding protein (MID), a 200-kDa outer membrane protein comprising 2,139 amino acids, has recently been isolated and shown to display a unique and specific affinity for human IgD. To identify the IgD-binding region, MID was digested with proteases. In addition, a series of truncated fragments of MID were manufactured and expressed in Escherichia coli followed by analysis for IgD binding in Western and dot blots. The smallest fragment with essentially preserved IgD binding was comprised of 238 amino acid residues (MID\(^{962-1200}\)). Shorter recombinant proteins gradually lost IgD-binding capacity, and the shortest IgD-binding fragment comprising 157 amino acids (MID\(^{985-1142}\)) displayed a 1,000-fold reduced IgD binding compared with the full-length molecule. The truncated MID\(^{962-1200}\) was efficiently attracted to a standard IgD serum and to purified myeloma IgD(κ) and IgD(λ) sera but not to IgG, IgM, or IgA myeloma sera. Furthermore, the fragment specifically bound to peripheral blood B lymphocytes, and the binding was inhibited by preincubation with anti-IgD-Fab polyclonal antibodies. Results obtained by introducing five amino acids randomly into MID\(^{962-1200}\) using transposons suggested that α-helix structures were important for IgD binding. Ultracentrifugation experiments and gel electrophoresis revealed that native MID\(^{962-1200}\) was a tetramer. Interestingly, tetrameric MID\(^{962-1200}\) attracted IgD more than 20-fold more efficiently than the monomeric form. Thus, a tetrameric structure of MID\(^{962-1200}\) is crucial for optimal IgD-binding capacity.

Nonimmune Ig-binding proteins derived from Gram-negative bacteria are rare compared with Ig-binding proteins from Gram-positive bacteria. However, Brucella abortus interacts with bovine IgM (1) and Haemophilus somnus with IgG-Fc (2), and some strains of Escherichia coli exhibit IgG binding in a nonimmune manner (3). In addition, Haemophilus influenzae and Moraxella catarrhalis have been shown to strongly bind human IgD (4). It has also been demonstrated that M. catarrhalis and H. influenzae activate human B cells through interactions with surface IgD (5). We have recently purified and characterized a 200-kDa outer membrane protein from M. catarrhalis designated Moraxella IgD-binding protein (MID)\(^1\) (6). The deduced amino acid sequence of MID consists of 2,139 residues. MID has specific IgD-binding properties and interacts with two purified human IgD myeloma proteins, four IgD myeloma sera, and one IgD standard serum. In contrast, no binding is detected to myelomas of other immunoglobulin classes including IgG, IgM, IgA, and IgE. MID also binds to the surface-expressed B cell receptor (BCR) IgD but not to other membrane molecules on human peripheral blood lymphocytes. Furthermore, MID is mitogenic for purified B cells and induces Ig production in the presence of Th2 cytokines (7).

IgD is a unique immunoglobulin, which was first discovered in 1965 by Rowe and Fahley (8). Because soluble IgD is easily degraded by proteolytic enzymes and therefore difficult to purify, the specific role of IgDs has not yet been completely defined (9). Soluble IgD represents approximately 0.25% of the total serum immunoglobulins. In contrast, cell surface-bound IgD serves as an antigen BCR and is co-expressed with IgM on mature B lymphocytes. Interestingly, an increased number of IgD molecules has been observed in human bronchus-associated lymphoid tissue and tonsils (10), suggesting a possible interaction between the immune system and the IgD-binding respiratory pathogens M. catarrhalis and H. influenzae (4, 6).

In the present study, we show that a recombinant protein corresponding to a 238-amino acid sequence between positions 962 and 1200 of MID essentially has a preserved IgD-binding capacity on a molar basis compared with the fully intact MID\(^{1-2139}\) molecule. The truncated protein formed a tetramer as revealed by gel electrophoresis and ultracentrifugation analyses. The tetrameric molecular bound IgD more than 20-fold efficiently compared with the monomeric counterpart.

MATERIALS AND METHODS

Reagents—M. catarrhalis B5 was a clinical isolate from a nasopharyngeal swab culture obtained from our department (6). E. coli DH5α or BL21(DE3) (Novagen; Darmstadt, Germany) were used for most transformations. For vectors containing fragments A and B, the host BL21(DE3)-pLysS (Novagen) was used. Transformed bacteria were grown in Luria Bertani broth supplemented with kanamycin. In the case of BL21(DE3)-pLysS, chloramphenicol was also added. The expression vector pET-26b(+) was from Novagen, and the pMAL expression vector was purchased from New England Biolabs (Beverly, MA). The

* This work was supported by grants from the Alfred Österlund Foundation, the Anna and Edwin Berger Foundation, the Crafoord Foundation, the Greta and Johan Kock Foundation, the IngaBritt and Arne Lundberg Foundation, the Magnus Bergvall Foundation, the Swedish Medical Research Council, the Swedish Society of Medicine, the Åke Wiberg Foundation, and the Cancer Foundation at the University Hospital in Malmö. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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† The abbreviations used are: MID, Moraxella IgD-binding protein; BCR, B cell receptor; PBL, peripheral blood lymphocyte; HRP, horseradish peroxidase; pAb, polyclonal antibody; mAb, monoclonal antibody; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; RPE, R-phycocerythrin.
human IgD myeloma whole sera IgD(e) and IgD(λ) were obtained from The Binding Site (Birmingham, England), and the IgD standard serum OTRD 02/03 was from Behringwerke (Marburg, Germany). Myeloma whole sera IgD, IgM, and IgA were included as described previously (6). Horseradish peroxidase (HRP)-conjugated goat anti-human IgD polyclonal and bovine serum albumin (BSA)-conjugated anti-human IgD, κ- and λ-light chain polyclonal antibodies were from Dakopatts (Glostrup, Denmark). To produce a specific anti-MID902–1200 antiserum, rabbits were immunized intramuscularly with 200 μg of purified recombinant protein emulsified in complete Freund’s adjuvants (Difco, Becton Dickinson; Heidelberg, Germany) and boosted on days 15, 30, and 60 with the same dose of protein in incomplete Freund’s adjuvants. Blood was drawn 2 to 3 weeks later. HRP-conjugated swine anti-rabbit Pab (Dakopatts) were used as detection antibodies. Antibodies used for flow cytometry were R-phycocyanin (RPE)-labeled anti-human CD19 (Dakopatts) mAb and the Ig-fraction of rabbit anti-human IgD-Fab (4), which was further purified by passing through a polyacrylamide- Sepharose column.

Peptide Cleavage of MID and Amino Acid Sequence Analysis—MID was isolated and purified from *M. catarrhalis* Bc5 as described previously (6). Native MID in 0.05 M Tris-HCl (pH 8.8) containing 0.1% Empigen® was digested with trypsin or chymotrypsin at an enzyme/protein ratio of 1:10 at 37 °C overnight or with Endoproteinase LysC at an enzyme/protein ratio of 1:50 (Sigma) at 30 °C overnight. The digestion mixtures were run on SDS-PAGE, and protein bands were then transferred electrophoretically to Immobilon-P membranes (Millipore, Bedford, MA) and analyzed for IgD binding. The fragments were sequenced with an Applied Biosystems ( Foster City, CA) 470A gas-liquid-solid-phase sequenator (11). The resulting sequences were for trypsin TAAQANTESSIAGV, GNTANFSVNSGDDALN, and QGINDENAAFVRLGEK, and for chymotrypsin PSTVKA D. When MID was digested with LysC, the sequence MID535–1172 was obtained by mass spectrometry (12).

DNA Cloning and Protein Expression—All truncated MID constructs were manufactured using PCR-amplified fragments. Tag DNA polymerase was from Roche Molecular Biochemicals, and PCR conditions were as recommended by the manufacturer. The open reading frame of the mid gene from *M. catarrhalis* (pET26b(MID)) was used as template (6). All MID constructs, except for MID927–960 (fragment C), were amplified by PCR using specific primers introducing the restriction enzyme sites BamHI and HindIII. Because fragment C has an internal HindIII site, an XhoI was used instead of HindIII at the 3′-end. The PCR products were cloned into pET28a (+), except for fragment I, which was cloned into pMAL-c2. To avoid presumptive toxicity, the resulting plasmids were first transformed into host E. coli BL21 (DE3) and then transferred electrophoretically to Immobilon-P membranes (Millipore, Bedford, MA) and analyzed for IgD binding. The fragments were sequenced with an Applied Biosystems ( Foster City, CA) 470A gas-liquid-solid-phase sequenator (11). The resulting sequences were for trypsin TAAQANTESSIAGV, GNTANFSVNSGDDALN, and QGINDENAAFVRLGEK, and for chymotrypsin PSTVKD. When MID was digested with LysC, the sequence MID535–1172 was obtained by mass spectrometry (12).

Ultracentrifugation—Sedimentation equilibrium analysis was performed in a Beckman Optima XL-A analytical ultracentrifuge equipped with absorbance optics, using an An50Ti rotor. MID927–1200 was equilibrated in PBS buffer, whereas 0.1 M NH4HCO3 containing 0.5% Empigen® was used for native purified MID. Short column experiments were performed at two consecutive speeds (9,000 and 11,000 rpm). After sedimentation equilibrium was reached, absorbance scans were taken with 0.001-cm step size, 8 averages, at appropriate wavelengths (231, 236, 280, or 286 nm). Six-hole centerpieces of charcoal-filled Epon (12-nm optical path) were used, and the equilibrium temperature was 20 °C. High-speed sedimentation at 48,000 rpm was conducted afterward for baseline correction. Estimates of the buoyant molar mass of the protein were determined by fitting a sedimentation equilibrium model for a single sedimenting solute to individual data sets with the program EQASSOC (13). These values were converted to the corresponding average molar masses using the partial specific volume calculated from the amino acid composition of the protein (14). Several models of self-association were globally fitted to multiple sedimentation equilibrium using the MULTSEQ program, kindly supplied by D. Crothers, Boston, National Institute of Health, Bethesda, MD.

Mutation Generation Using Transposons—Five extra amino acids were randomly inserted into the IgD-binding region of MID (fragment F2, MID927–1200) using the Mutation Generation System (Finnzymes, Espoo, Finland) according to the manufacturer’s instructions. Because the pET28a (+) vector has an internal NdeI site, MID927–1200 was first subcloned into pUC18 (CLONTECH, Palo Alto, CA) using the restric-
tion enzyme sites BamHI and HindIII. The artificial transposon Entronposon was inserted with an in vitro transposition reaction catalyzed by a single purified MuA transposase. The transposition reaction proceeded for 1 h at 30 °C followed by transformation of HB101 (Bio-Rad) by electroporation. Since the Entronposon codes for chloramphenicol resistance the clones could easily be selected. After plasmid preparation (Qiagen, Hilden, Germany), the insert was digested with BamHI and HindIII and separated by agarose gel electrophoresis. The inserts containing the Entronposon were further cloned into pUC18 followed by transformation into DH5α. The resulting clones were pooled, and the plasmid DNA was extracted. The Entronposon was removed by digestion with NotI leaving 15 base pairs. DNA was ligated and the plasmids transformed into BL21(DE3). The resulting clones containing five extra amino acids at different positions were tested for specific IgD-binding capacity using colony-blots. Nitrocellulose blotting membranes (Sartorius; Göttingen, Germany) were incubated on the plates with E. coli clones. After 20 min, bacteria on the membranes were lysed with chloroform treatment for 30 min. The membranes were washed, blocked, and incubated with IgD as described above for Western blots. After amino acid sequencing, individual positions in MID could be discerned (Fig. 1A). Trypsin digestion resulted in a 40-kDa IgD-binding band and 35- and 80-kDa bands that did not interact with IgD. Furthermore, chymotrypsin treatment resulted in, inter alia, a 25-kDa non-IgD-binding band, whereas digestion with LysC gave, among others, a 66-kDa IgD-binding band. These initial experiments thus indi-
cated that an IgD-binding region was located approximately between amino acid residues MID864 and MID1172.

IgD Binding Is Preserved in 238 Amino Acids of MID

To determine in more detail the MID IgD-binding region, 11 sequences derived from the full-length MID were cloned and expressed in E. coli. The recombinant proteins covered the entire MID sequence, and the individual lengths and positions were as shown in Fig. 1. The recombinant proteins comprising amino acid residues 69–1111 (void of the signal peptide) or 1011–2139 of MID did not bind IgD, whereas all other fragments bound IgD. DNA encoding for the various truncated MID proteins were cloned into the expression vector pET28a+(+) and produced in E. coli. The recombinant proteins containing histidine tags were purified and dot blotted onto a nitrocellulose membrane. The membrane was probed with human IgD followed by secondary HRP-conjugated polyclonal antibodies that were used for detection.

Fig. 2. MID962–1200 (fragment F2) has a conserved IgD-binding capacity compared with full-length MID1–2139. Equimolar concentrations (ranging from 240 to 0.06 nmol) of purified full-length MID1–2139 and eight truncated MID fragments (F1–F8) were analyzed for IgD binding by dot blots. The proteins MID962–1130 (F6), MID965–1120 (F7), and MID1000–1200 (F4) did not attract IgD, whereas all other fragments bound IgD.

To pinpoint the sequence responsible for the IgD binding, the truncated MID902–1200 was systematically shortened at the N- and C-terminal ends (Fig. 2). Equimolar concentrations of the various recombinant proteins were compared with native full-length MID1–2139 isolated from M. catarrhalis. The different recombinant proteins were diluted in 4-fold steps, added to membranes, and incubated with human IgD. On a molar basis, an essentially preserved IgD-binding capacity was detected for the truncated MID protein stretching from amino acid residue 962 to 1200. The shortest truncated protein still interacting with IgD was localized between MID985 and MID1142 (fragment F6). The IgD binding property was lost when the N terminus was reduced to the MID1000 residue (fragment F4) or when the C-terminal was shortened to MID1130 (fragment F7). Finally, fragment F8 (MID902–1130), with a longer N-terminal and a shorter C-terminal (compared with MID965–1200, fragment F3), was also manufactured and analyzed. However, this truncated MID did not interact with IgD, suggesting that the binding capacity was depending on a longer C-terminal.

To further characterize the specific MID-dependent IgD binding, an ELISA was constructed using human IgD as bait.
All of the recombinant truncated MID fragments were subjected to ELISA followed by incubation with a specific rabbit anti-serum directed against MID902. The ELISA was developed using HRP-conjugated goat anti-rabbit polyclonal antibodies. The same pattern as with the dot blot (Fig. 2) was observed, i.e. fragments F4, F7, and F7 were not attracted to the solid-phase IgD, whereas the other fragments bound to a variable degree compared with full-length MID (not shown).

**Native MID and the Truncated MID962-1200 Are Tetramers as Shown by Sedimentation Equilibrium Analysis and Gel Electrophoresis**—In a previous publication, we estimated with chromatography and SDS-PAGE that native MID is aggregating and exists as an oligomer (6). To confirm these results, a sedimentation equilibrium analysis on MID was performed. Purified MID, which was solubilized in the detergent Empigen® (0.5%), was subjected to gradient centrifugation at two different speeds in an ultracentrifuge supplied with absorbance optics. An apparent molar mass of ~1,000 kDa was obtained, and it is concluded that native MID forms an oligomer.

In addition to full-length MID, we subjected the truncated MID962-1200 (F2) to a sedimentation equilibrium analysis. The data obtained were compared with the calculated values for a tetramer or dimer. The experimental gradient for MID962-1200 protein (27 μM) as well as the theoretical ones for dimer and tetramer species are shown in Fig. 3A. The predominant MID962-1200 species sedimented at equilibrium with an average molecular mass of 110 ± 3 kDa. This was compatible with a protein tetramer because MID962-1200 including a histidine tag has a molecular mass of 27 kDa as calculated from the amino acid composition. To analyze the putative quaternary structure of MID962-1200 (F2) for IgD binding, the recombinant protein was subjected to analysis in gel electrophoresis under native conditions. In addition, a corresponding IgD Western blot analysis was performed. MID962-1200 migrated as a protein with an apparent molecular mass of ~100 kDa in the native gel (Fig. 3B), confirming the ultracentrifugation experiments. Thus, in addition to the sedimentation equilibrium analysis, the experiment with the native gel suggested that MID962-1200 in its native form was a tetramer consisting of four molecules. Furthermore, using native conditions, the tetrameric structure of MID962-1200 strongly bound IgD (Fig. 3B).

**Optimal MID962-1200-IgD Interaction Is Dependant on Tetramer Structure**—To further shed light upon the need for a tetramer structure to obtain an optimal IgD binding, MID962-1200 (F2) was incubated at 60 or 100 °C followed by analysis on SDS-PAGE and Western blots. MID962-1200 formed both a monomer and a tetramer after pretreatment at 60 °C (Fig. 4A). The tetrameric structure was, however, dis
ruptured at 100 °C and resulted in a monomeric form, which displayed a considerably weaker binding to IgD when examined in Western blots (Fig. 4, A and B). To investigate the capability of the tetramer to bind IgD in comparison with the monomeric form, the MID962-1200 fragment was subjected to analysis at 60 °C in six different experiments. The heat-treated protein was subjected to SDS-PAGE, and the IgD binding activity was analyzed by Western blots. Resulting gels and filters were analyzed by densitometry, and the protein concentration (density) of the monomer was divided with the corresponding tetramer concentration. The obtained value (%) was related to the concentration (µg) of total protein loaded on the gels. Interestingly, when IgD binding to the tetrameric respectively monomeric forms were compared, a 23-fold more efficient binding to IgD was found with the tetrameric MID962-1200 (Fig. 4C).

The Specific IgD Binding of MID962-1200 (F2) Includes α-Helix Structures—The MID962-1200 sequence was subjected to computer analysis (Fig. 5A). A possible secondary structure of the truncated MID including four α-helices and several β-strands was observed. Data based upon sequence analysis suggested that a reduction in the number of amino acids at the N- and C-terminals of MID962-1200 would thus destroy the secondary structure, causing a weaker IgD binding that would eventually disappear. Therefore, five amino acid residues were randomly introduced into MID962-1200 using transposons and a MuA transposase in vitro. A fraction of the resulting E. coli clones (n = 90) were examined for IgD binding, and their MID962-1200-containing plasmids were sequenced. Two different groups were consequently found, i.e. binding and non-binding clones (Fig. 5B). When the sequence MID962-1200 was interrupted with the five extra amino acids, IgD binding was completely abolished. Another sequence (MID1100-1145) that was upstream of the molecule was also found to be important for IgD binding. Interestingly, those sequences included α-helix structures. In contrast, MID962-1200 still attracted IgD when five extra amino acid residues were introduced near the N-terminal region or between MID1059 and MID1100, i.e. two sequences not containing α-helices. Insertion of extra amino acids within the end of the C-terminal-flanking region did not considerably influence IgD binding, a finding that was partly consistent with the truncated MID fragments shorter than MID962-1200. Computer analysis revealed that when five amino acid residues were introduced into sequences responsible for α-helix formation, that particular structure was disrupted. Thus, the secondary structure analysis (Fig. 5A) compared with the data obtained with transposons confirmed the results seen with the truncated MID protein series (Fig. 2).

MID962-1200 (F2) Specifically Binds to Human IgD and Is Attracted to the IgD BCR—Full-length MID1-2139 attracts human IgD but not immunoglobulins from the other subclasses (6). To verify the specificity of the interaction between IgD and recombinant MID962-1200, a dot blot experiment was performed. The membrane was coated with the truncated MID protein followed by incubation with IgD, IgG, IgM, or IgA myeloma sera. As shown in Fig. 6, MID962-1200 interacted with the three IgD myeloma sera, whereas MID962-1200-dependent binding was not detected to myeloma sera representing the IgG, IgM, or IgA subclasses.

The next step was to investigate whether truncated MID also was attracted to membrane-bound IgD, i.e. the IgD BCR. MID962-1200 was conjugated to FITC followed by incubation with purified PBLs. In parallel, cells were labeled with RPE-conjugated anti-CD19 monoclonal antibodies. After washes, PBLs were subjected to flow cytometry analysis. Interestingly,
FITC-conjugated MID$^{962–1200}$ significantly bound to the CD19$^+$ B lymphocyte population (Fig. 7B). To ensure that MID$^{962–1200}$, FITC specifically interacted with the IgD BCR, PBLs were also preincubated with anti-human IgM-Fab polyclonal antibodies. As seen in Fig. 7C, the MID$^{962–1200}$-FITC-dependent IgD binding was completely abolished when the IgD BCR was blocked by anti-IgD-Fab PAbs. These results were in agreement with the previously published data on full-length MID (6).

**DISCUSSION**

The novel outer membrane protein MID from *M. catarrhalis* recently has been identified and shown to display a strong and specific affinity for human IgD (6). In the present paper, we use proteolytic fragments of MID and recombinantly expressed proteins to demonstrate that the IgD binding was essentially preserved in a truncated recombinant protein located between amino acid residues 962 and 1200 of MID (Fig. 2). The molecular mass of the smallest protein still binding IgD comprised 157 amino acids. The IgD-binding capacity of the recombinant proteins was related to individual lengths, *i.e.* the binding decreased dramatically with the length. This may be because the binding is highly dependant on a specific molecular structure of the IgD-binding region. This hypothesis was further strengthened by our ultracentrifugation and gel electrophoresis experiments. We have shown previously that native MID isolated from *M. catarrhalis*, in addition to the 200-kDa band, forms a band that seemingly is an oligomer with a molecular mass of $\sim$1,000 kDa in SDS-PAGE (6). When purified MID solubilized in the detergent Empigen$^®$ (0.5%) was analyzed by ultracentrifugation, an apparent molecular mass of $\sim$1,000 kDa was observed. This molecular mass was consistent with a tetramer of MID in the presence of Empigen$^®$. The smallest truncated MID protein (MID$^{962–1200}$, fragment F2) with an essentially preserved IgD binding activity (compared with full-length MID) and which could be analyzed by ultracentrifugation in the absence of detergent was shown to be a tetramer under native conditions. Interestingly, when tetrameric and monomeric forms of MID$^{962–1200}$ were separated by SDS-PAGE and analyzed for IgD binding, the tetramer was shown to attract IgD $>20$-fold more efficiently compared with the monomeric form. Thus, the tetrameric structure was considerably more active compared with the monomer. We cannot fully exclude, however, that the monomeric protein was completely unreactive because it may refold to a tetrameric structure during the blotting process.

The MID region required for IgD binding is large compared with other bacterial surface proteins that bind immunoglobulins. Nonimmune Ig binding activity was first observed in *Staphylococcus aureus* with the discovery of protein A that attracts IgG (16). Protein A interacts with the Fc part of IgG, but only with subclasses 1, 2, and 4 (17). In addition, protein A binds a fraction of Ig molecules of all classes because of specific structural features (18). Protein A is a 45-kDa protein composed of five extracellular domains that form a putative microfibril. Each of the extracellular domains contains 240–250 amino acid residues and is rich in glycine (18). The initial hypothesis for the binding of protein A is that it contains a common structure for all IgG subclasses, including IgG1, IgG2, and IgG3. However, studies have shown that protein A interacts with the Fc part of IgG, but only with subclasses 1, 2, and 4 (17). In addition, protein A can also bind IgM, IgA, and IgD (19). Protein A interacts with the Fc part of IgG, but only with subclasses 1, 2, and 4 (17). In addition, protein A can also bind IgM, IgA, and IgD (19).

In conclusion, we have shown that the IgD-binding region of the MID protein comprises a 238-amino acid long sequence that is not repeated in MID. Furthermore, tetramer formation is of fundamental importance for optimal IgD binding.

**Acknowledgments—** We thank Marta Brant for excellent technical assistance, Drs. Germán Rivas and Carlos Alfonso (Servicio de Ultracentrifuga Analítica, Centro de Investigaciones Biológicas, Madrid, Spain) for ultracentrifugation analyses, and Drs. Bo Ek and Håkan Larsson (Swedish University of Agriculture Sciences, Uppsala, Sweden) for mass spectrometry analyses.

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