Recognition of Mesothelin by the Therapeutic Antibody MORAb-009

**STRUCTURAL AND MECHANISTIC INSIGHTS**

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**Background:** Mesothelin is a tumor differentiation antigen; its binding to tumor antigen CA-125 can lead to tumor metastasis.

**Results:** Structures of the Fab from a therapeutic antibody MORAb-009 and its complex with an epitope-containing fragment of mesothelin are obtained.

**Conclusion:** Overlapping binding sites for both CA-125 and MORAb-009 provides a basis for the antibody therapeutic effect.

**Significance:** This work represents the first experimental structure for mesothelin.

Mesothelin is a cell surface protein that is normally found in mesothelial cells lining the pleura, pericardium, and peritoneum (1, 2) but is aberrantly expressed at a high level in a variety of cancers including mesothelioma, ovarian, pancreatic, and lung cancers (3–8). The human mesothelin gene (MSLN gene) encodes a 69-kDa precursor protein that is subsequently processed by the endoprotease furin to yield a 40-kDa glycosyl-phosphatidylinositol-anchored mesothelin (Msln)3 (2) and a 31-kDa megakaryocyte-potentiating factor (9) (Fig. 1A). Although the physiological function of mesothelin is unclear, studies have shown that it is capable of binding to the tumor antigen CA-125 (also known as MUC16) and mediates cell adhesion (10–12). CA-125 is a well documented biomarker for ovarian cancers (13), and the majority (88%) of mesothelioma cases are also CA-125-positive on the cell membrane (14), suggesting the possibility that binding of tumor-associated CA-125 to mesothelin on normal mesothelial cells lining the pleura or peritoneum can lead to heterotypic cell adhesion and tumor metastasis within the pleural and peritoneal cavities. By truncation and alanine replacement mutagenesis, the CA-125 binding site was mapped to a 64-residue fragment at the N terminus of mesothelin (12).

The basis for anti-mesothelin cancer therapy is the observation that levels of antibodies specific for mesothelin are elevated in the sera of patients with mesothelioma and epithelial ovarian cancer and that this elevation is associated with high expression of mesothelin in tumors (15). Antibody response to mesothelin-expressing ovarian carcinoma cells may be responsible for reduction of tumor load and contribute to prolonged survival (16). Because mesothelin is specifically expressed at a significantly higher level in malignant tumors, development of an antibody against mesothelin is, therefore, of major importance in the field of cancer therapy (17). MORAb-009 is a promising antibody with potential clinical applications currently under-
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Going Phase II clinical trials. It is a chimeric IgG1/κ antibody that was generated by fusing the genes encoding the anti-mesothelin Fv (SS1 scFv) in-frame with human IgG1 and κ constant regions (17). Animal experiments have shown that application of MORAb-009 or its conjugate with pseudomonas exotoxin A in combination with chemotherapy leads to a marked reduction in tumor growth of mesothelin-expressing tumors (18, 19). Clinical studies demonstrated that it blocks the binding of mesothelin to CA-125 and thus could be used as a strategy to prevent tumor metastasis (20).

The potential application of MORAb-009 goes beyond its direct binding to mesothelin. Its Fv fragment is being tested as a carrier to deliver various anticancer agents to target cells. An anti-mesothelin recombinant immunotoxin, SS1-PE38 or SS1P, composed of the Fv portion of MORAb-009 (SS1) and a truncated form of *Pseudomonas* exotoxin (PE38) (21), was developed and evaluated in clinical studies (7). Despite this significant progress, an understanding at the atomic level of the interaction of MORAb-009 with the CA-125 antigen is still lacking. Here, we report the crystal structures of both the antigen-free Fab fragment of MORAb-009 and its complex with an N-terminal fragment of mesothelin.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of Full-length Wild-type and Triple Mutant Mesothelin**—Full-length cDNA of mesothelin was inserted into the baculovirus transfer vector pAcGP67B of BD BaculoGold™ (BD Biosciences) in-frame with the hexahistidine tag at the C terminus. All mutations were made by PCR using the QuikChange™ mutagenesis kit (Agilent Technologies, Inc., Wilmington, DE). The plasmid was co-transfected with linearized viral DNA into ~2 million Sf9 cells, and the culture was gradually amplified to 10 liters of cultured insect cells for secretory expression of mesothelin. Culture media was collected and concentrated in a diafiltration device (Millipore, Billerica, MA) against a diafiltration solution containing 10 mM imidazole. After washing with the column. After washing the column with the washing buffer, the bound protein was eluted by the same buffer containing 300 mM imidazole. Fractions containing mesothelin were pooled and concentrated. Mesothelin was further purified by gel filtration using a Superdex 75 column equilibrated with 20 mM Tris, pH 8.0, 100 mM NaCl.

**Blue Native PAGE (BN-PAGE) Analysis of Mesothelin-Fab Complex**—The purified full-length wild-type or triple mutant mesothelin in 25 mM Tris, pH 7.5, 200 mM NaCl was mixed with MORAb-009 Fab and incubated at room temperature for 30 min to allow formation of the complex. The complex was then subjected to BN-PAGE analysis following the procedure as described in Ma and Xia (26).

**Preparation of Fab Fragment from IgG—MORAb-009 IgG was obtained from Morphotek, Inc. (Exton, PA). Fab fragment was prepared using the Fab preparation kit from Thermo Scientific (Rockford, IL) and following the instructions from the manufacturer.**
was solved by the molecular replacement (MR) method using the BALBES (28) program in the CCP4 program suite (29) and subsequently refined using Phenix (30) and REFMAC (31). The crystal structure of the complex of Fab and Msln-(7–64) was determined also by MR using MOLREP (32) and refined with REFMAC. All structure models were built using the program COOT (33). The atomic coordinates have been deposited in the Protein Data Bank as follows: PDB ID code 4F33 for the Fab fragment derived from MORAb-009 and 4F3F for the complex between Fab and mesothelin N-terminal fragment.

RESULTS

Identification of the Minimal Fragment Containing the Epitope Recognized by MORAb-009—Fully processed, full-length mesothelin has 303 amino acid residues, starting from the precursor protein Glu-296 to Asp-598 (Fig. 1A). For convenience, we renumber the residues in mature mesothelin starting with Glu-1 and ending with Asp-303. Full-length mesothelin with two additional C-terminal residues followed by a hexahistidine tag (the lower panel) was expressed for analysis. The three N-glycosylation sites in mesothelin (Asn-93, Asn-193, and Asn-220) are indicated.

Previously, it was reported that a 64-residue N-terminal fragment of mesothelin is the minimal size for CA-125 interaction and that this interaction can be blocked by the monoclonal antibody SS1 Fv (12) from which MORAb-009 is derived. We constructed a vector to express in E. coli the 64-residue fragment of mesothelin fused C-terminally to TrxA. We found by Western blot that the purified fusion protein was highly reactive to MORAb-009. Using this construct, we made a series of C-terminal truncates in approximately 10-residue decrements (Fig. 2C, top panel). The results showed that mesothelin N-terminal fragments up to 50 residues in length did not react with MORAb-009. When the size was increased to 59 residues or longer, the antibody binding was restored. To further narrow down the boundaries that define the minimal fragment for binding, the protein was truncated from the N terminus, and the results showed that the minimal mesothelin fragment to react with MORAb-009 is between residues 7 and 59. Further truncation from the N terminus led to production of fragments non-reactive to the antibody (Fig. 2C, bottom panel). However, the $K_d$, as measured by isothermal titration calorimetry, of this N-terminal fragment of mesothelin indicated a 5-fold reduction in binding affinity to the antibody (data not shown).
An alignment of available mesothelin sequences from mammals shows that the two cysteine residues (Cys-7 and Cys-31) in the N-terminal fragment are conserved, indicating a possible disulfide bond (Fig. 1B). To test the presence of this disulfide bridge, we mutated residue Cys-7 to serine. Indeed, the mutation abolishes its ability to bind MORAb-009 (Fig. 2C, lower panel), underlining the importance of this disulfide linkage in maintaining the structural integrity of mesothelin.

**Structure Determination for the Fab-Mesothelin Complex and Isolated Fab**—To investigate the interaction between mesothelin and MORAb-009 at atomic resolution, we made a new construct expressing the minimal fragment from residue Cys-7 to Leu-64 and fused a hexahistidine tag at its C terminus (Msln-(7–64)). This fragment was expressed and purified and then mixed with the Fab fragment from MORAb-009. The resulting complex was purified by size-exclusion chromatography and crystallized. Crystals of the Msln-(7–64)-Fab complex diffracted x-rays to better than 2.5 Å resolution and a data set processed to 2.61 Å resolution was obtained (Table 1). To facilitate the structure determination of the complex by the MR method using the structure of the antibody as a phasing template, we needed to crystallize the Fab fragment alone and determine its structure first.

The structure of isolated MORAb-009 Fab was solved by MR using a template Fab structure (PDB code 1A6T) from a neutralizing monoclonal antibody against human rhinovirus 14 and was refined to 1.75 Å resolution (Table 1). There are four Fab fragments in a crystallographic asymmetric unit; each fragment includes 212 residues (2–213) for the light chain and 220 residues (1–220) for the heavy chain. The four non-crystallographic symmetry-related Fab fragments showed nearly identical conformation, with root mean square deviations from pair-wise superposition ranging from 0.167 to 0.434 Å using all 432 Ca atoms. Therefore, we used in all subsequent analysis the fragment containing chain E (light chain) and chain F (heavy chain), which had the best overall electron density.

The coordinates of the MORAb-009 Fab fragment were used as a search model to solve the structure of the complex of Fab and Msln-(7–64). The resulting MR phases allowed calculation of difference Fourier maps that revealed additional electron densities attributable to residues of the N-terminal fragment of mesothelin. The atomic model for the structure of the complex was refined to 2.6 Å resolution (Table 1, Fig. 3A) and consists of 211 residues from the light chain (2–212), 220 residues from the heavy chain (1–220), and 59 residues from mesothelin (6–64), including an extra N-terminal residue, Met-6. The electron density for residues in the range 135–138 of the heavy chain is not well defined probably due to a high degree of flexibility. Residue Thr-51 of the light chain of MORAb-009 is an outlier in Ramachandran plot, which persisted in all five independently determined Fab structures reported here regardless of whether it is in complex with the antigen or not. This residue is part of the cdr2 that has no contact with the antigen; it is located in a tight turn and appears to be conformationally rigid.

**The Structure of Mesothelin N-terminal Fragment**—The structure of mesothelin N-terminal fragment represents the first experimental model for mesothelin and its mammalian homologs. The structural model of Msln-(7–64) consists of an N-terminal loop (residues 7–17) followed by five consecutive α-helices named sequentially as α1 (18–22), α2 (25–31), α3 (34–39), α4 (41–46), and α5 (51–63), which are linked by short loops (Figs. 1B and 3A and B). These secondary structural elements spiral into a right-handed, two-turn superhelix; helices α2 and α5 are nearly parallel to each other. The structure is stabilized by a disulfide bridge between the N-terminal Cys-7 and Cys-31 at the end of helix α2, which has well defined electron density (Fig. 3C) and is consistent with both the mutagenesis data (Fig. 2C) and the observed sequence conservation for these two residues (Fig. 1B). The N-terminal fragment of meso-
Mesothelin possesses a dipole moment roughly running along the axis of the superhelix. Searching an existing protein structure data base did not identify any structure with significant structural similarity.

Interactions between Fab and Mesothelin—As shown in Fig. 3A, the complex is formed with binding contacts from both the light and heavy chains of Fab, although the majority of interactions are contributed from the heavy chain. The epitope recognized by MORAb-009, as revealed by the structure, consists of two non-consecutive antigenic determinants; the first is centered on helix α1 and extends on both sides from Glu-18 of the N-terminal loop to the beginning of helix α2 (Trp-26). The second contains the loop between helices α4 and α5 (Fig. 3, A and B). On the opposite side of the mesothelin epitope are residues from complementarity-determining regions (CDRs) of the Fab, forming a slightly concave surface that surrounds the epitope. The numbering of the Fab sequence in this work follows the convention used in a previous publication (17).

TABLE 1
Statistics on x-ray diffraction data sets and refined structural models

| Data set   | Fab          | Msn-(7–64)-Fab |
|------------|--------------|---------------|
| Wavelength (Å) | 1.0          | 1.0           |
| Space group | P4_2_2       | P4_2_2        |
| Cell dimension (Å) | a = b = 140.6, c = 282.0, α = β = γ = 90 | a = b = 146.2, c = 80.9, α = β = γ = 90 |
| Resolution (Å) | 39.1-1.75 (1.81-1.75) | 50.0-2.61 (2.72-2.61) |
| No. of observations | 1,436,883 | 186,593 |
| No. of unique reflections | 262,387 | 29,264 |
| Redundancy | 5.5 (2.1) | 6.4 (2.4) |
| Completeness (%) | 92.0 (70.7) | 97.8 (80.0) |
| Rmerge (%) | 9.2 (40.7) | 10.1 (44.2) |
| Mean I/σ(I) | 19.5 (1.4) | 22.4 (1.8) |

Refinement

| No. of reflections | Working set | 229,079 | 27,763 |
|--------------------|-------------|---------|--------|
|                   | Test set    | 2,630   | 1,485  |
|                   | Rcryst (%)  | 20.2    | 18.9   |
|                   | Rmerge (%)  | 22.7    | 23.5   |
| Residues          | Heavy chain | 880     | 216    |
|                   | Light chain | 848     | 211    |
|                   | Mesothelin  | 0       | 59     |
|                   | Water       | 1,328   | 47     |
|                   | PEG         | 6       | -      |
|                   | Average B-factor (Å²) | 32.3 | 56.8 |
| Root mean square deviations | Bond length (Å) | 0.007 | 0.019 |
|                   | Bond angle (°) | 1.1    | 2.2    |
| Ramachandran plot statistics | Most favored region | 1346 (90.9%) | 370 (87.3%) |
|                   | Allowed regions | 130 (8.8%) | 53 (12.5%) |
|                   | Disallowed region | 4 (0.3%) | 1 (0.2%) |

* Statistics on x-ray diffraction data sets and refined structural models.

* Rmerge is defined as Σ|Ii| - ⟨I⟩)/ΣIi, where Ii is the intensity for ith observation of a reflection with Miller index h, and ⟨I⟩ is the mean intensity for all measured Ii's and Friedel pair.

Conformational Adaptation in Fab upon Binding to Mesothelin—A structure superposition of the isolated Fab with that in complex with mesothelin gave a root mean square deviation of 0.346 Å over 359 Ca atoms demonstrated a globally stable conformation for the Fab in two different crystal envi-
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![Diagram](image-url)

**FIGURE 3. Molecular interactions between mesothelin and Fab.** A, shown is a ribbon diagram of the structure of the Msln-(7–64)-Fab complex. The mesothelin fragment is colored in magenta. The N-terminal disulfide bond is illustrated as sticks in yellow. The Fab light chain and heavy chain are colored in cyan and blue, respectively. Variable and constant domains of the light chain (V_{L} and C_{L}) and the heavy chain (V_{H} and C_{H}) are indicated. CDRs of the light chain are labeled as L1, L2, and L3, respectively, and those of heavy chain are labeled H1, H2, and H3, respectively. Inset, shown is an enlarged view of the hydrophobic and aromatic-aromatic interactions between Phe-22 of mesothelin and Fab. Residues involving in interacting with Phe-22 of mesothelin and Fab. Residues undergoing large conformational changes upon bind-}

It was previously reported that the cancer antigen CA-125 recognizes the N-terminal region of mesothelin and the recognition site is conformationally sensitive (12). The monoclonal...
antibody SS1, the predecessor of MORAb-009, is capable of interrupting CA-125 binding to mesothelin. In this work we demonstrate unambiguously by truncation, cysteine mutagenesis, and crystallographic experiments that the antigenic epitope for the monoclonal antibody MORAb-009 is also nonlinear and conformationally sensitive (Figs. 2 and 3). The epitope is bipartite, consisting of residues from helix H9251 and a loop between helices a4 and a5 and has a footprint of 870 Å².

Mechanism of Disruption of CA-125 Binding to Mesothelin by MORAb-009—Although the physiological function of mesothelin remains obscure, its N-terminal portion appears particularly immunogenic. In particular, the N-terminal part has been shown to be responsible for CA-125 binding, suggesting a likely route for cancer metastasis. Site-directed mutagenesis has demonstrated that residue Tyr-23 of mesothelin is critical for CA-125 binding; residues Trp-26 and Glu-29 may also play a role, whereas His-59 is not involved in binding (12). Mapping these residues onto the structure of the mesothelin and Fab complex, we are able to show that the CA-125 binding region partially overlaps with the binding epitope of MORAb-009 and centers perhaps on the loop between helices a1 and a2 (Fig. 4C). In the complex structure, residue Tyr-23 of mesothelin is mostly buried by the surrounding residues of mesothelin and by CDR L3 of Fab, with only the -OH group exposed to the surface. In the free form, however, the Tyr-23 would most likely be exposed to the surface and is accessible for interacting with CA-125 or with the antibody. Thus, MORAb-009 exerts its action to block attachment of CA-125 by competing for binding to the same region, making Tyr-23 inaccessible for CA-125 interaction. Both residues Trp-26 and Glu-29 of mesothelin are constituents of helix a2 and have exposed side chains in the complex. Although the side chain of Trp-26 does form an H-bond with the hydroxyl group of Tyr-32 of the Fab light chain, Glu-29 does not have any contact with the antibody. Thus the partially destroyed CA-125 binding activity by mutating either residue indicates proximity of these residues to the binding site. Residue His-59 is on the other side of the putative CA-125 binding site, consistent with the mutagenesis data that its mutation does not interfere with CA-125 binding.

Correlation of Mesothelin Binding Energy with Specific Mutations on MORAb-009—The efficacy of therapeutic antibodies or immunotoxins has been shown to correlate with their binding affinities and, for solid tumors, also with their sizes. Using the phage display affinity maturation technique, mutations were introduced to residues on the L3 CDR loop of the parental antibody of MORAb-009, namely the mouse antibody SS (17). A series of mutations were identified that showed improved affinity for mesothelin and cytotoxicity to cultured mesothelin-expressing cancer cells (Table 2). Different from its parental line, MORAb-009 or SS1 bears two mutations (G93K and Y94H) that give rise to a significantly higher affinity for mesothelin.
thelin with a $K_d$ of 0.72 nM over the parental $K_d$ of 11 nM (Table 2), as measured by the surface plasmon resonance method. The structure shows that both residues are part of the promorphic niche that embraces the side chain of Phe-22 of mesothelin. Although Lys-93 provides its main chain atoms to the pocket, His-94 uses both main- and side-chain atoms. His-94 additionally forms an H-bond with Ser-59 of the heavy chain; both His-94 and Ser-59 join forces to interact with mesothelin Glu-18 with distances of 3.5 and 2.6 Å, respectively, for His-94 and Ser-59 of the heavy chain.

The atomic details of the Fab-Msln-(7–64) complex provide the structural basis for changes in affinities for other variants. Mutants S92G/G93F/Y94N (D8), S92G/G93S/Y94H (C10), and L96T (E4) showed higher affinity with $K_d$ values of 0.3, 0.2, and 3 nM, respectively (Table 2). From the structure (Fig. 3A, inset), all these mutations altered the binding pocket to various extents for Phe-22 of mesothelin. Residue Phe-93 in D8 may interact with Tyr-23 of mesothelin. Residue Gly-92 in both D8 and C10 probably makes the L3 loop more flexible to promote a conformational change for better interactions with Phe-22 of mesothelin. The residue Leu-96 forms the floor of the Phe-22 binding niche, and mutation L96T clearly alters the interaction by providing an additional dipolar interaction between the phenyl ring of Phe-22 and the hydroxyl group of Thr-96.

It should be pointed out that based on the structure of the Msln-(7–64)-Fab complex, we estimated relative free energies for binding of mesothelin by various SS variants, and the antibody SS1, which is equivalent to MORAb-009, clearly stands out as having the lowest free energy for binding to mesothelin (Table 2). It did not escape our notice that all previous efforts in improving the antibody concentrated on the L3 loop. Our structural data show, on the contrary, that the majority of interactions between mesothelin and antibody are in fact provided by CDRs of the heavy chain, optimization of which could provide further improvement for the affinity of antibody toward mesothelin.

**Homologous Proteins and Molecular Modeling Studies of Mesothelin**—The mesothelin superfamily of proteins, which includes mesothelin, mesothelin precursor, megakaryocyte potentiating factor, stereocilin, and otoancorin were predicted to have superhelical structures with Armadillo or Armadillo-type repeats (25). Functionally, these repeats have been suggested to interact with carbohydrate moieties of extracellular glycoproteins (25). The structure of the N-terminal 64 residues reported here is indeed superhelical even though a detailed comparison showed large deviations.

**TABLE 2**

| Antibody | Mutations          | $K_d$, nM | Cytotoxicity | Estimated relative free energy, kcal/mol |
|----------|--------------------|-----------|-------------|----------------------------------------|
| SS*      | Ser-92, Gly-93, Tyr-94, Leu-96 | 11        | 1           | −110                                   |
| SS1*     | G93K, Y94H         | 0.72      | 13          | −131                                   |
| D8       | S92G, G93F, Y94N   | 0.3       | 11          | −100                                   |
| C10      | S92G, G93S, Y94H   | 0.2       | 11          | −75                                    |
| E4       | L96T               | 3         | 2           | −93                                    |
| E4       | L96T               | 3         | 2           | −93                                    |

a Data were obtained from the Ref. 17.

b Free energies for the mesothelin-Fab complex and for mesothelin and Fab alone were estimated in the program Phenix without refinement. The energy difference between individual components and the complex was taken as the estimated relative free energy upon antigen-antibody association.

c SS, parental mouse monoclonal antibody.

d SS1, MORAb-009.

e A model of MORAb-009 binding to mesothelin displayed on the cell surface. Based on the previously published full-length mesothelin model, a hybrid model was constructed by replacing the N-terminal fragment of mesothelin with the experimental structure Msln-(7–64). The C terminus is anchored to the cell membrane through a glycosylphosphatidylinositol (GPI) linker.
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