A Binding Domain on Mesothelin for CA125/MUC16*

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Ovarian cancer and malignant mesothelioma frequently express both mesothelin and CA125 (also known as MUC16) at high levels on the cell surface. The interaction between mesothelin and CA125 may facilitate the implantation and peritoneal spread of tumors by cell adhesion, whereas the detailed nature of this interaction is still unknown. Here, we used truncated mutagenesis and alanine replacement techniques to identify a binding site on mesothelin for CA125. We examined the molecular interaction by Western blot overlay assays and further quantitatively analyzed by enzyme-linked immunosorbent assay. We also evaluated the binding on cancer cells by flow cytometry. We identified the region (296–359) consisting of 64 amino acids at the N-terminal of cell surface mesothelin as the minimum fragment for complete binding activity to CA125. We found that substitution of tyrosine 318 with an alanine abolished CA125 binding. Replacement of tryptophan 321 and glutamic acid 324 with alanine could partially decrease binding to CA125, whereas mutation of histidine 354 had no effect. These results indicate that a conformation-sensitive structure of the region (296–359) is required and sufficient for the binding of mesothelin to CA125. In addition, we have shown that a single chain monoclonal antibody (SS1) recognizes this CA125-binding domain and blocks the mesothelin-CA125 interaction on cancer cells. The identified CA125-binding domain significantly inhibits cancer cell adhesion and merits evaluation as a new therapeutic agent for preventing or treating peritoneal malignant tumors.

Ovarian cancer largely is confined to the peritoneal cavity for much of its natural history (1). Peritoneal mesothelioma is a highly invasive tumor originating from the mesothelial linings of the peritoneum (2). The development of effective drug regimens against ovarian cancer and mesothelioma has proven extremely difficult. Mesothelin was first identified in 1992 by the monoclonal antibody (mAb) K1 that was generated by the immunization of mice with human ovarian carcinoma (OVCAR-3) cells (3). The mesothelin gene encodes a 71-kDa precursor protein that is processed to a 40-kDa protein termed mesothelin, which is a glycosylphosphatidylinositol (GPI)-anchored glycoprotein present on the cell surface (4). Mesothelin is a differentiation antigen that is present on a restricted set of normal adult tissues such as the mesothelium. In contrast, it is overexpressed in a variety of cancers including mesothelioma, ovarian cancer, and pancreatic cancer (5). In addition, mesothelin is also expressed on the surface of non-small cell lung cancer cells (6, 7), especially most lung adenocarcinomas (8).

We and others have shown that mesothelin is shed from tumor cells (9, 10), and antibodies specific for mesothelin are elevated in the sera of patients with mesothelioma and ovarian cancer (11). Shed serum mesothelin has been approved by the United States Food and Drug Administration (FDA) as a new diagnostic biomarker in mesothelioma. In a Phase I clinical study of an intrapleural interferon-β gene transfer using an adenoviral vector in patients with mesotheliomas, we found that antitumor immune responses targeting mesothelin were elicited in several patients (12). A recent study indicated that anti-mesothelin antibodies and circulating mesothelin relate to the clinical state in ovarian cancer patients (13). Pastan and colleagues (14) developed an immunotoxin (SS1P) with a Fv for mesothelin. Two Phase I clinical trials were completed at the National Cancer Institute (National Institutes of Health, Bethesda, MD) and there was sufficient antitumor activity of SS1P to justify a Phase II trial. A chimeric antibody containing the mouse SS1 Fv for mesothelin was also developed and is currently examined in a Phase I clinical trial for ovarian cancer, mesothelioma, pancreatic cancer, and non-small cell lung cancer (15).

Mucins are heavily glycosylated proteins found in the mucus layer or at the cell surface of many epithelia (16). There are two structurally distinct families of mucins, secreted and membrane-bound forms. CA125 (also known as MUC16) was first identified in 1981 by OCV125, a mAb that had been developed from mice immunized with human ovarian cancer cells (17). The first cDNA clones were reported in 2001 (18, 19). CA125 is a very large membrane-bound cell surface mucin, with an average molecular mass between 2.5 and 5 million daltons. It is also heavily glycosylated with both O-linked and N-linked oligosaccharides (20). The peptide backbone of CA125 is composed of the N-terminal region, extensive Ser/Thr/Pro-rich tandem repeats (TR) with 156 amino acids each with both N- and O-glycosylated assay; f, rabbit Fc; hMSLN, human mesothelin; scFv, single chain variable fragment; mnMSLN, mouse mesothelin; PE, phycoerythrin; HEK, human embryonic kidney.
Mesothelin and CA125/MUC16

cosylations, a SEA domain with high levels of O-glycosylation and a C-terminal region with a short cytoplasmic tail (19). The SEA domain was first identified as a module commonly found in sea urchin sperm protein, enterokinase and agrin (21, 22). The significance of the SEA domain in CA125 is not clear.

CA125 was originally used as a biomarker in ovarian cancer due to its high expression in ovarian carcinomas and that it is shed into the serum (23). A majority (88%) of mesotheliomas are also CA125 positive on the cell membrane (24). It was shown that 25% of peritoneal mesotheliomas have high CA125 expression (25). The intensity of CA125 membranous expression is indistinguishable between ovarian carcinomas and peritoneal mesotheliomas. Gene expression analysis using the SAGE tag data base has shown that mesothelioma has the second highest co-expression of CA125 and mesothelin after ovarian cancer (26). Rump and colleagues (26) have shown that 25% of peritoneal mesotheliomas have high CA125 expression (25). The intensity of CA125 membranous expression is indistinguishable between ovarian carcinomas and peritoneal mesotheliomas. Gene expression analysis using the SAGE tag data base has shown that mesothelioma has the second highest co-expression of CA125 and mesothelin after ovarian cancer (26). Rump and colleagues (26) have shown that 25% of peritoneal mesotheliomas have high CA125 expression (25). The intensity of CA125 membranous expression is indistinguishable between ovarian carcinomas and peritoneal mesotheliomas.

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Here, we identified the binding site of CA125 on mesothelin by use of truncated mutagenesis and alanine replacement approaches. We measured binding qualitatively by Western blot overlay assays and quantitatively by enzyme-linked immunosorbent assay (ELISA). We also evaluated the interaction of CA125 and mesothelin on cancer cells by flow cytometry. Furthermore, we have shown that a single chain mAb (SS1) recognized the CA125-binding domain and blocked the mesothelin-CA125 interaction on cancer cells. The identified CA125-binding domain-Fc fusion protein also significantly inhibited cancer cell adhesion. Our results suggest that conformation-sensitive structures of the region (296–359) are required and sufficient for specific binding of mesothelin to CA125. The domain proteins or the antibodies that block the mesothelin-CA125 interaction merit evaluation as new therapeutic agents in treating peritoneal malignant tumors.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**OVCA-3 (ovarian) cells were grown in RPMI 1640 (Dulbecco) supplemented with 20% fetal bovine serum, 1% penicillin/streptomycin, 1% L-glutamine, and 0.2% human insulin. You (mesothelioma) cells were grown in RPMI 1640 (Dulbecco) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% L-glutamine. HEK 293T cells were grown in 100-mm tissue culture dishes (Falcon) with Dulbecco’s modified Eagle’s medium and supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% L-glutamine.

**Truncated Mutant Constructs—**Full-length and fragments of mesothelin were amplified by PCR from PMH107 (GenBank™ accession number AF318239) (29). Primers were designed to incorporate flanking EcoRI and NotI restriction enzyme sites to facilitate in-frame cloning into a modified pSecTag2 vector (Invitrogen) (Table 1). Constructs contained an Igκ leader sequence followed by the rabbit IgG Fc and the full-length sequence of the extracellular domain of mesothelin (PMH113) or its fragments, followed by a Myc epitope and His6 tag. The rabbit IgG Fc (rFc) and mesothelin fragments were separately by a thrombin cleavage site and a flexible linker. The rFc-mouse

**TABLE 1**

| Prims used to construct truncated mutants of mesothelin |
|----------------------------------------------------------|
| The restriction enzyme sites are underlined.            |
| **Fragments** | **Prims (5’→3’)** |
| Region I | Forward: AGAAGAAGAAATCTGAGGAGGAAGAGACGGCTTCTT |
| Region II | AGAAGAAGAAATCTGAGGAGGAAGAGACGGCTTCTT |
| Region III | AGAAGAAGAAATCTGAGGAGGAAGAGACGGCTTCTT |
| Region IBC | Reverse: GTTTTCTTCTGCGGCCGCGAGCTCATCCAGTTTAGCCTTTAGGACGT |
| Region IAB | Reverse: GTTTTCTTCTGCGGCCGCGAGCTCATCCAGTTTAGCCTTTAGGACGT |
| Region IA | Reverse: GTTTTCTTCTGCGGCCGCGAGCTCATCCAGTTTAGCCTTTAGGACGT |
| Region IBC | Reverse: GTTTTCTTCTGCGGCCGCGAGCTCATCCAGTTTAGCCTTTAGGACGT |
| Region IB | Reverse: GTTTTCTTCTGCGGCCGCGAGCTCATCCAGTTTAGCCTTTAGGACGT |
| Region IC | Reverse: GTTTTCTTCTGCGGCCGCGAGCTCATCCAGTTTAGCCTTTAGGACGT |
| Region ICRegion IC | Reverse: GTTTTCTTCTGCGGCCGCGAGCTCATCCAGTTTAGCCTTTAGGACGT |

**TABLE 2**

| Prims used to generate alanine replacement mutants of mesothelin |
|---------------------------------------------------------------|
| The restriction enzyme sites are underlined.                  |
| **Mutants** | **Prims (5’→3’)** |
| Y318A | Forward: AGAAGAAGAAATCTGAGGAGGAAGAGACGGCTTCTT |
| W321A | Reverse: CTTTCTTCTGCGGCCGCGAGCTCATCCAGTTTAGCCTTTAGGACGT |
| E324A | Reverse: CTTTCTTCTGCGGCCGCGAGCTCATCCAGTTTAGCCTTTAGGACGT |
| F344A | Reverse: CTTTCTTCTGCGGCCGCGAGCTCATCCAGTTTAGCCTTTAGGACGT |
| E347A | Reverse: CTTTCTTCTGCGGCCGCGAGCTCATCCAGTTTAGCCTTTAGGACGT |
| K353A | Reverse: CTTTCTTCTGCGGCCGCGAGCTCATCCAGTTTAGCCTTTAGGACGT |
| H354A | Reverse: CTTTCTTCTGCGGCCGCGAGCTCATCCAGTTTAGCCTTTAGGACGT |
| K355A | Reverse: CTTTCTTCTGCGGCCGCGAGCTCATCCAGTTTAGCCTTTAGGACGT |

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mesothelin fusion (pMH117) was constructed using the same strategy. Alanine mutants were generated by PCR mutagenesis of pMH107-derived human mesothelin (hnMSLN) cDNA followed by cloning into the modified pSecTag2 vector. Mutations were introduced in either forward or reverse primers or by using a two-step overlap-extension PCR (Table 2).

Transfections and Mesothelin Mutant-Fc Fusion Protein Production—HEK 293T cells were grown until 60% confluent on 100-mm tissue culture dishes (Falcon). Constructs encoding mesothelin-Fc fusion proteins were transiently transfected using Lipofectamine (Invitrogen) in 6 ml of serum-free media. Three to 5 h later, 6 ml of 20% fetal bovine serum, Dulbecco’s modified Eagle’s medium was added to each dish and incubated for 48 h. Media was harvested subsequently on a daily basis and replaced with fresh media. Fc fusion proteins were purified from the media using columns containing Protein A-Sepharose (Amersham Biosciences). One-ml columns were loaded, washed with citrate/phosphate buffer, pH 5.0, and eluted with 100 mM glycine-HCl, pH 3.0, and neutralized in 1 M Tris, pH 8.0. Fractions were collected using the AKTA FPLC system (GE Healthcare) and pooled and concentrated. Final protein concentration was measured using Coomassie Plus Protein Assay Reagent (Pierce). Fractions of the dominant peak were run on a SDS-PAGE gel under non-reducing and reducing conditions. To verify that generated proteins were not aggregated, mesothelin and its mutants were subsequently run over a TSK gel filtration size exclusion column (TOSOH Bioscience LLC, Montgomeryville) at 0.5 ml/min in phosphate-buffered saline, pH 7.5.

Flow Cytometry—to determine binding of mesothelin fragments to CA125 on the cell surface, OVCAR-3 or YOU cells were grown until confluent, detached, and then incubated with 1 µg/ml of mesothelin or its fragments in FACS buffer (5% bovine serum albumin, 0.01% NaN₃) for 1 h on ice. Bound fragments were detected by incubating with a 1:200 dilution of goat anti-rabbit IgG-PE (BIOSOURCE) secondary antibody in FACS buffer for 0.5 h on ice. Cells were analyzed using FACSCalibur (BD Biosciences). Each binding experiment was repeated three to five times.

In inhibition assays, cells were incubated with FLAG-tagged mesothelin and an excess amount (10-fold) of mesothelin or mesothelin fragments without a FLAG tag for 1 h on ice. Bound FLAG-tagged mesothelin proteins were detected by incubating with 1:100 dilution of an anti-FLAG tag mAb (Sigma) followed by PE-conjugated goat anti-mouse IgG (BIOSOURCE).

Sandwich ELISA—Nunc MaxiSorp 96-well flat-bottomed plates were incubated overnight with 5 µg/ml goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) in phosphate-buffered saline, followed by an overnight block with 5% bovine serum albumin, 0.01% NaN₃ in phosphate-buffered saline. Purified Fc mesothelin fragments were diluted to 1 µg/ml in ELISA buffer (0.01% Tween 20, 10% Pierce SuperBlock) and incubated on a plate for 1 h at room temperature. Plates were then incubated with OVCAR-3 supernatant containing CA125 for 1 h at room temperature. To detect bound CA125 a 1:200 dilution of

FIGURE 1. Generation of truncated and alanine replacement mutants of mesothelin. A, the mesothelin (MSLN) gene encodes a precursor protein of 622 amino acids. On translocation into the endoplasmic reticulum the N-terminal signal peptide (red, residues 1–33) and the C-terminal GPI anchor addition signal (blue, a predicted cleavage site: Ser598) are removed and the latter replaced with a GPI anchor. The MSLN precursor (71-kDa) is cleaved into two products, the 30-kDa megakaryocyte potentiating factor (MPF; residues Ser34–Arg286) (31) and the 41-kDa GPI-anchored membrane-bound mature MSLN (orange) starting from Glu296. The proteolytic cleavage region (green) contains a furin cleavage site at Arg295, and other protease cleavage sites including a trypsin cleavage site at Arg286. The four predicted N-linked glycans (black lollipops; Asn57, Asn388, Asn488, and Asn515) on mesothelin are indicated. Truncated mutants (Regions I, II, III, IAB, IBC, IA, IB, and IC) were generated as rabbit Fc fusion proteins to sequentially narrow down the CA125-binding domain of mesothelin. B, mesothelin and truncated mutants were generated using a modified pSecTag2 vector that when transfected into HEK 293T cells created secreted rabbit Fc fusion proteins. A CMV promoter (P_CMV) drove the expression of an Ig-k signal, followed by a rabbit Fc fragment, a thrombin cleavage site, and the desired portion of mesothelin. A His₆ tag was added at the C-terminal of the construct.
anti-CA125 mAb (Zymed Laboratories Inc.) was incubated for an additional hour at room temperature; subsequently a 1:1500 dilution of goat anti-mouse IgG horseradish peroxidase conjugate (BIOSOURCE) was added for 1 h at room temperature. The plates were washed four times with ELISA buffer between each coating. Visualization was achieved with 3,3′,5,5′-tetramethylbenzidine detection reagent (KPL) and absorbance was read at 450 nm with SpectraMax Plus plate reader (Molecular Devices).

**Western Blots**—Purified Fc mesothelin fragments (500 ng) were mixed with Laemmli sample buffer (Bio-Rad) supplemented with 5% β-mercaptoethanol. Samples were boiled for 2 min and separated on 4–20% SDS-PAGE gels (Invitrogen). After transfer for 4 h at 30 V, the polyvinylidene difluoride membrane was blocked overnight at 4 °C in 1% Western Blocking Reagent (Roche) in Tris-buffered saline (50 mM Tris-HCl, 150 mM NaCl). The membrane was then incubated with OVCAR-3 supernatant for 1 h at room temperature. This was followed by incubation with 1:200 dilution of OC125 mAb for 1 h at room temperature. Detection was performed with goat anti-mouse IgG horseradish peroxidase conjugate (BIOSOURCE) at 1:1000 for 1 h.

**Determination of Affinity Constants** (K_D) As previously described (29), equilibrium constants and Scatchard plots were determined by using the Marquardt-Levenberg algorithm for nonlinear regression with Prism software (version 5.0, GraphPad Software, San Diego, CA).

**Mammalian Cell Display**—As previously described (30), single chain Fv antibody (scFv) SS1 was cloned into an expression vector (pMH112) for cell surface expression of scFv on HEK 293 cells.

**Heterotypic Cancer Cell Adhesion Assay**—We followed a recently developed protocol (15). Briefly, OVCAR-3 or YOU cells (1 x 10^5) were seeded in triplicate in microplates, incubated overnight at 37 °C, in 5% CO2. The following day, H9 (A431-Mesothelin +) (11) were harvested and loaded with Calcein AM cell dye (Invitrogen) as per the instruction manual. The OVCAR-3 or YOU monolayers were washed once with 200 μl of 10% complete RPMI and preincubated for 1 h at 4 °C with purified mesothelin Fc fusion proteins. The labeled H9 (2 x 10^5) cells were added to triplicate wells for 1 h at 4 °C. Wells were gently washed five times with 200 μl of phosphate-buffered saline by inverting the plate on paper towels. Cell adherence and its inhibition were quantitated using VICTOR3 Multilabel Counter model 1420 (PerkinElmer Life Sciences).

**Statistical Analysis**—The data obtained were entered in Prism (version 5) for Windows (GraphPad Software) for statistical analysis. Flow cytometry raw data were analyzed by analysis of variance with Dunnett’s and Newman-Keuls multiple comparison post tests. p values < 0.05 were considered statistically significant.

**RESULTS**

**Generation of Mesothelin Mutants**—Truncated mutants of mesothelin were generated to sequentially narrow down the binding domain to CA125. As shown in Fig. 1, portions of mesothelin were PCR amplified to incorporate NotI and EcoRI restriction sites and cloned into a modified pSecTag 2B vector containing an N-terminal rFc fragment. HEK 293T cells were transfected and Fc mesothelin proteins were collected and purified from the supernatant over the course of 8–10 days. Constructs encoding amino acid residues 296–390 (Region I), 391–486 (Region II), and 487–581 (Region III) of mesothelin were initially generated and tested. Constructs encoding smaller fragments within Region I, Region IAB (296–359), Region IBC (328–405), Region IA (296–337), Region IB (328–369), and Region IC (360–405) were also generated. It was

![FIGURE 2. Key residues proposed for alanine mutations. A, OVCAR-3 cells were incubated with 1 μg/ml of human mesothelin (hnMSLN) or mouse mesothelin (mnMSLN) rFc fusion proteins. The mesothelin binding on OVCAR-3 cells was detected by a goat anti-rabbit IgG Fc PE conjugate. B, eight residues identical between hnMSLN and mnMSLN in Region IAB were selected for alanine replacement. Region IAB starts at Glu296 and ends at Leu359 (*) in hnMSLN. Alkaline mutants within Region IAB were expressed. Alkaline mutants Y318A, W321A, E324A, and H354A (arrows) were secreted and purified for analysis. The other four mutants, F344A, E347A, K353A, and K355A, were not secreted and found aggregated inside transfected HEK 293T cells.](image-url)
found that secretion of the IA (296–337) fragment was prevented by protein aggregation; however, this problem was circumvented by moving the rFc to the C terminus of the mesothelin fragment.

PCR mutagenesis was used to generate a panel of alanine mutants within region 296–359 (Region IAB). As shown in Fig. 2A, both human and mouse mesothelin (mmMSLN) proteins bind human CA125. It was reasoned that those residues conserved between the two species would be more likely to be involved in the interaction. Specific amino acids were targeted based on the homology between human and murine mesothelin (Fig. 2B). In addition, we hypothesized that these residues are likely to be involved in the carbohydrate binding. In total, eight alanine mutant constructs were made: Y318A, W321A, E347A, K353A, H354A, and K355A. Of these mutants, four were secreted at high enough levels to purify using a Protein A column, Y318A, W321A, E324A, and H354A. The purity and molecular weight of each purified protein was confirmed on SDS-PAGE. The other four mutants, F344A, E347A, K353A, and K355A, were not secreted into the culture supernatants due to aggregation inside cells according to immunoblotting of whole cell lysates (data not shown), indicating that mutations of these residues may cause misfolding of mesothelin. For all proteins purified, a distinct peak was found on a TSK size exclusion column (Fig. 3). The rFc-extracellular portion of mesothelin-(296–581) fusion protein (full-length) was estimated to be ~75 kDa, whereas truncated mutants were relatively smaller in molecular mass (~50 kDa for Regions I, II, and III and ~40 kDa for IAB, IBC, and alanine mutants of IAB).

**Binding of CA125 to Mesothelin and Its Mutants**—To examine the interaction of CA125 and mesothelin mutants, we used Western blot overlay analysis. We ran equal amounts (500 ng) of each protein on an SDS-PAGE gel and transferred the protein to a polyvinylidene difluoride membrane. Membrane was blotted with OVCAR-3 supernatant containing CA125 followed by OC125, an anti-CA125 mAb. As shown in Fig. 4, the full-length extracellular domain of mesothelin (296–581), Region I (296–390), and Region IAB (296–359) bound CA125. A 64-amino acid fragment (Region IAB) at the N terminus of mesothelin (296–359) retained 100% binding capability to CA125. However, three smaller fragments, Region IA (296–337), Region IB (328–369), and Region IC (360–405), consisting of ~42 amino acids covering all the residues within Region I showed no binding to CA125 in Western blot. The alanine mutation at His^354_ did not change the mesothelin-CA125 interaction. Interestingly, alanine mutations at Tyr^318_ (Y318A) and Glu^324_ (E324A)
binding of the Fc fusion protein of wild-type mature mesothelin (296–598), Region I (296–390), and IAB (296–359) to CA125 was ~3 nM. The \( K_D \) for the mesothelin-CA125 interaction is consistent with the value (~5 nM) previously obtained on OVCAR-3 cells by flow cytometry (28). Three smaller fragments (296–337, 328–369, and 360–405) within Region I showed no binding to CA125 in ELISA (data not shown), indicating the first 64 residues at the N terminus of cell surface mesothelin is the irreducible binding domain on mesothelin for the CA125 protein. It is striking that substitution of the tyrosine at position 318 with an alanine (Y318A) completely disrupted the interaction with CA125. Alanine mutations at Glu324 (E324A; \( K_D = 42.4 \text{ nM} \)) and Trp321 (W321A; \( K_D = 19.5 \text{ nM} \)) reduce the binding of mesothelin to CA125. The alanine mutation at His354 (H354A) does not change the mesothelin-CA125 interaction (\( K_D = 2.71 \text{ nM} \)).

To verify that fragments were specifically binding to CA125, an ELISA was employed wherein captured mesothelin fragments were incubated with either OVCAR-3 supernatant containing CA125 or supernatant from the OVCAR-3 with knockdown of CA125 expression. No signal was detected from those fragments incubated with the supernatant from CA125-knockdown cells (data not shown), indicating that the binding between mesothelin and CA125 in ELISA was specific.

**Cell Binding Assays by Flow Cytometry**—To assess binding to CA125 on cancer cells, wild-type mesothelin and its mutants were incubated with cells. As shown in Fig. 6, Region I (a 95-amino acid fragment consisting of residues 296–390 at the N terminus) of mesothelin was found to bind to OVCAR-3 cells, whereas fragments Region II (391–486) and Region III (487–581) showed no binding. The smallest fragment that still contained most (~90%) of binding activity to CA125 was Region IAB. Three smaller fragments within Region I, Region IA (296–337), IB (328–369), and IC (360–405), were also tested. Only Region IB had modest (~10%) CA125-binding activity. These

FIGURE 5. Binding kinetics of mesothelin mutants and CA125. Scatchard plots (left) were made. ELISA plates captured the Fc mesothelin mutant fusion proteins at various concentrations (x axis; see "Experimental Procedures"). OVCAR-3 supernatant containing soluble CA125 was then added, followed by the OC125 mAb and a goat anti-mouse IgG horseradish peroxidase. Visualization was achieved with 3,3′,5,5′-tetramethylbenzidine detection reagent and absorbance was read at 450 nm (y axis). The full-length mature form of mesothelin (FULL) bound to CA125 with an approximate affinity of 1.54 nM. Region IAB wild-type (wt) (296–359) had a \( K_D \) of 3.35 nM. Substitution of the tyrosine at position 318 with an alanine (Y318A) completely disrupted the interaction with CA125. Alanine mutation at Glu324 (E324A; \( K_D = 42.4 \text{ nM} \)) and Trp321 (W321A; \( K_D = 19.5 \text{ nM} \)) reduce the binding of mesothelin to CA125. The alanine mutation at His354 (H354A) does not change the mesothelin-CA125 interaction (\( K_D = 2.71 \text{ nM} \)).
data indicate that IAB, the first 64 residues at the N terminus of cell surface mesothelin, is the minimum region that retains the most binding activity to CA125. The IAB-binding domain was found to bind with comparable affinity, when compared with the full-length mesothelin (FULL). This suggests that it is primarily the N terminus of cell surface mesothelin that is involved in the interaction with CA125 and the minimum sequence for CA125 binding activity is Region IAB (328–405).

Four alanine mutants (Y318A, W321A, E324A, and H354A) generated within the region 296–359 were similarly assessed for their ability to bind to CA125 on the surface of OVCAR-3 cells. It was found that the substitution of alanine for tyrosine at residue 318 completely ablated binding (Fig. 6). The H354A mutant conversely showed comparable binding to CA125 on the surface of OVCAR-3 cells.

Using analysis of variance with Dunnett’s and Newman-Keuls multiple comparison post tests, we have demonstrated
that full-length mesothelin (FULL), Region I, IAB, and the H354A mutant significantly bound to CA125 on OVCAR-3 cells, as compared with Regions II, III, IBC, IA, IB, IC, and the Y318A, W321A, and E324A mutants ($p < 0.05$). As compared with Region IAB, the binding by full-length mesothelin (FULL), the H354A mutant, and Region I was not significant ($p < 0.05$), indicating that IAB is the minimal sequence of CA125-binding domain.

Because surface protein expression of mesothelin and CA125 are also found frequently in malignant mesothelioma, we then examined the binding of mesothelin and its mutants to the YOU mesothelioma cell line (Fig. 7). The binding patterns were similar to those seen in OVCAR-3 cells except for Region I. As shown in Fig. 7, wild-type mesothelin (FULL), Regions I and IAB bound CA125 on YOU cells significantly stronger than Regions II, III, and IBC ($p < 0.05$). Unlike what we saw in OVCAR-3 cells, Region I retained about 40% of the CA125 binding activity on YOU cells. Nevertheless, the binding of Region IAB to CA125 on YOU cells is comparable with the full-length mesothelin (FULL) ($p > 0.05$). We also found that Region IB had modest (~10%) binding activity. The alanine replacements at positions 318, 321, and 324 showed a significant decrease of CA125 binding activities (10–30%) on YOU cells.

Inhibition of the Mesothelin-CA125 Interaction by the CA125-binding Domain—The molecular mechanisms underlying the cell adhesion and signaling induced by the mesothelin-CA125 interaction are not clear. An ideal antagonist drug would disrupt the mesothelin-CA125 interaction but not induce cell adhesion and signaling. To this end, we examined if any of the mesothelin truncated or alanine mutants reported here can compete with the binding of wild-type mesothelin to CA125. We co-incubated a FLAG-tagged wild-type mesothelin with a panel of our mesothelin mutants (Fig. 8). Region IAB can effectively inhibit the mesothelin-CA125 interaction. Other constructs such as Region I and H354A can also inhibit the mesothelin-CA125 interaction, whereas Regions II, III, and IBC, mutants Y318A, E324A, and W321A cannot. We have demonstrated that full-length mesothelin (FULL), Region I, IAB, and the H354A mutant significantly inhibited the binding.
of mesothelin with a FLAG tag to CA125 on OVCAR-3 cells, as
compared with Regions II, III, IBC, and the Y318A, W321A, and
E324A mutants (p < 0.05). As compared with Region IAB, the
inhibition of the methelin-CA125 interaction by full-length
mesothelin (FULL) and Region I was not significant (p > 0.05),
clearly indicating that IAB is the minimal sequence (296–359)
of the CA125-binding domain.

Epitope Mapping of scFv SS1 by Mammalian Cell Display—
Because Fv SS1-derived antibody drugs are currently in several
multi-center clinical trials to treat mesothelioma and ovarian
cancer, we decided to examine its epitope using the mesothelin
fragments we have made. To this end, we used a new method
(called “mammalian cell display”) recently developed in the labo-
rary (30) to express the SS1 scFv on HEK 293 cells. We incubated
the HEK 293 cells expressing scFv SS1 with mesothelin and its
fragments or mutants. As shown in Fig. 9, the epitope of scFv SS1
overlaps the CA125-binding site on mesothelin.

Inhibition of the Mesothelin-CA125 Interaction by the SS1
scFv—We investigated whether or not the SS1 scFv could block
the mesothelin-CA125 interaction. We made the SS1 in the
format of a scFv SS1-PE38 fusion protein (8). We co-incubated
the SS1 or HA22, a scFv-PE38 specific for CD22 (29) with mesothelin
and OVCAR-3 or YOU cells. As shown in Fig. 10, the SS1 scFv
remarkably inhibited the mesothelin-CA125 interaction, whereas
the HA22 scFv did not.

Inhibition of Cancer Cell Adhesion by the CA125-binding Do-
main—Finally we explored the pos-
sibility that the CA125-binding
domain can functionally block can-
cer cell adhesion. We used the assay
system recently established by
Hassan et al. (15). We measured
adhesion of fluorescently labeled
mesothelin-expressing H9 cells (11)
onto CA125 positive OVCAR-3 or
YOU cancer cells. Fig. 11 shows the
results of this study. Significant het-
rotypic cell binding was seen on a
monolayer of OVCAR-3 or YOU
cells with the control CD22-Fc
fusion protein or a control frag-
ment (Region IBC). The CA125-binding
domain (IAB)-Fc fusion protein
completely abolished H9 cell adhe-
sion onto OVCAR-3 or YOU

cells. A statistically significant
inhibition with the CA125 domain was
detected with concentrations as low
as 1 μg/ml (Fig. 11).

Structure of CA125-binding Do-
main—Because a three-dimen-
sional structure of mesothelin is
currently not available, the sec-
ondary structure was evaluated by
the

algorithms PROF (predictprotein.org) and APSSP2 (imtech.
res.in/raghava/apssp2). The CA125-binding domain is primar-
ily composed of helix-turn-helix repeats (Fig. 12). Interestingly,
Tyr318 whose alanine replacement significantly reduces the
CA125 binding is located at the coil between two helical sec-
ondary structures. The tyrosine seems to be a critical residue
that either directly binds the N-glycan on CA125 or indirectly
plays an important role by maintaining a conformation
required for CA125 binding. The partial loss of CA125 binding
activities of the W321A and E324A mutants may indicate that
other residues near 318 are also involved. The fact that Region
IB alone had only a modest CA125-binding activity (~10%) in-
dicates that the CA125-binding domain requires Region IA
for its full activity.

DISCUSSION
Cancer cells commonly spread within the peritoneal cavity
via seeding to mesothelium-lined structures. The interaction
between CA125, a mucin present on a majority of ovarian can-
cer and mesothelioma cells, and mesothelin, a GPI-anchored
glycoprotein present on the mesothelial cells lining along the

FIGURE 11. The CA125-binding domain blocks the mesothelin/CA125-mediated cancer cell adhesion.
OVCAR-3 (A and C) or YOU cancer cells (B and D) formed monolayers. The OVCAR3 or YOU cancer cell monolayer
was preincubated with full-length mesothelin, the CA125-binding domain (IAB), IBC, or CD22 Fc fusion pro-
teins before fluorescently labeled mesothelin-expressing H9 cells were added. Mesothelin or IAB significantly
blocked cancer cell adhesion with concentrations as low as 10 μg/ml on OVCAR3 cells (A and C) or 1 μg/ml on
YOU cells (B and D) as compared with the control protein (CD22 or IBC) (*, p < 0.05).

Mesothelin and CA125/MUC16
mesothelin to allow binding to occur. Alanine mutants of Trp^{321} and Glu^{324} in the vicinity of the tyrosine at 318 also showed decreased binding to CA125 in all assays but were not nearly as dramatic. Mutating the histidine at 354 had no effect on the mesothelin-CA125 interaction.

During the preparation of SDS-PAGE, proteins are typically reduced and denatured by treatment with the Laemmli sample buffer. Many protein functions depend on protein secondary and tertiary structures, which could be disrupted under reducing and denaturing conditions. The fact that CA125 bound to mesothelin (296–581), Region I (296–390), and IAB (296–359) in Western blot overlay assays may indicate that the mesothelin proteins were renatured rapidly during the polyvinylidene difluoride membrane transfer. The renaturing may allow mesothelin to recover their secondary and tertiary structures specific for CA125 binding.

Mesothelin is predicted to be primarily composed of helix-turn-helix repeats. Interestingly, Tyr^{318} whose alanine replacement abolishes the CA125 binding of IAB is located at the coil between two helical secondary structures (Fig. 12), indicating that binding could be dependent on the conformation of this region.

We further showed that the CA125-binding domain (IAB) or its alanine mutant could effectively block the binding of wild-type mesothelin to CA125, indicating the CA125-binding domain Fc-fusion protein is a good candidate to be used as a potential antagonist to inhibit the mesothelin-CA125 interaction. Moreover, the Fc portion of the molecule with antibody-dependent cell-mediated cytotoxicity or complement-dependent cytotoxicity could recruit NK cells or complements to kill the CA125-positive tumor cells. Because of Enbel (TNFR-Fc), an FDA-approved Fc fusion drug, many peptide-Fc fusion proteins are currently in clinical trials (32).

Furthermore, we have shown that a single chain mAb (SS1) recognizes the CA125-binding domain and blocks the mesothelin-CA125 interaction on cancer cells. Most interestingly, the CA125-binding domain-Fc fusion significantly inhibits heterotypic cancer cell adhesion. The SS1 Fv-derived immunotoxin and a chimeric (the SS1 mouse Fv/human Fc) antibody are currently in clinical trials for mesothelioma, ovarian cancer, pancreatic cancer, and non-small cell lung cancer.

Here, we have shown that mesothelin binds to CA125 primarily with the N-terminal portion of cell surface mesothelin. The CA125-binding domain of 64 residues, likely a helical structure, is irreducible. We have further shown that a tyrosine at position 318 is critical for binding to CA125. The CA125-binding domain significantly inhibits cancer cell adhesion and merits evaluation as a new therapeutic agent in preventing or treating peritoneal malignant tumors.

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