Expression of a Recombinant Breast Tumor-associated Mucin Fusion Protein in Escherichia coli Exposes the Tumor-specific Epitope

Peisheng Hu1 and Stephen E. Wright2

Department of Internal Medicine [P. H., S. E. W.] and Biochemistry and Molecular Biology [S. E. W.], Texas Tech University Health Sciences Center, and the Department of Veterans Affairs Medical Center [S. E. W.], Amarillo, Texas 79106

ABSTRACT

Mucins are highly immunogenic glycoproteins that are abundantly expressed by breast carcinomas and other carcinomas. The fact that deglycosylated normal mucin can induce tumor-specific monoclonal antibodies indicates that tumor-specific epitopes are hidden in the fully glycosylated form. Using recombinant DNA techniques, a fragment of mucin tandem repeats was inserted into pMal-p, an Escherichia coli expression vector, and resulted in the expression of an unglycosylated maltose-binding protein-mucin fusion protein. This fusion protein has been purified and showed strong affinity to breast tumor-specific monoclonal antibody SM3. The antisera against this recombinant mucin fusion protein recognized all breast tumor cell lines we tested. Competition assay with monoclonal antibody SM3 shows that anti-deglycosylated fusion protein binds the epitope that SM3 binds. These results confirm the hypothesis that unglycosylated mucin contains a tumor-specific epitope. This leads to the possibility that recombinant mucin may be used to develop vaccines against breast cancer and cytotoxic T-lymphocyte lines for immunotherapy of breast cancer.

INTRODUCTION

Polymorphic epithelial mucins are high molecular weight (M, >250,000) and heavily O-linked glycosylated proteins. Many of the antigenic differences between carcinomas and adjacent normal epithelial tissues have been attributed to aberrant expression of these molecules. Altered expression of mucin has been reported for carcinomas of the breast (1–3), pancreas (4), ovary (5) and other organs (6). Mucin cDNAs4 from breast and pancreatic carcinomas show virtually identical sequence (7–9). The encoded protein of mucin cDNA consists of three distinct regions: the amino terminus consisting of putative signal peptide; the major portion of the protein consisting of only the tandem repeat region; and a unique region containing a transmembrane sequence with a cytoplasmic tail (7). Tandem repeats appear to be a general characteristic of mucin core proteins. Each tandem repeat consists of a 60-base pair nucleotide sequence encoding a 20-amino acid sequence. The genetic polymorphism of polymorphic epithelial mucins is due to differences in the number of tandem repeats. It appears that the mucins expressed by breast and other carcinomas are aberrantly glycosylated; the carbohydrate side chains of the cancer-associated mucin are shorter than that of the mucin produced by normal cells (10). This may result in the exposure of tumor epitopes which are hidden in the fully glycosylated form. A number of monoclonal antibodies raised by deglycosylated mucin have been shown to react selectively against tumor (11–13). One such monoclonal antibody, SM3, was shown to be tumor specific (11). This suggested that the deglycosylation of mucin results in the appearance of a new tumor-associated epitope.

In order to facilitate the study of tumor-associated epitopes and the practical use of the rMucin, a MBP-mucin hybrid protein was constructed in which seven tandem repeats of mucin core protein are fused to the COOH terminus of MBP from Escherichia coli. The fusion protein was expressed in E. coli in a nonglycosylated form. Presented here are the construction, expression, and characterization of this MBP-mucin chimeric protein as well as the properties of antisera from this recombinant fusion protein.

MATERIALS AND METHODS

Materials and Reagents. Restriction endonucleases and T4 ligase were purchased from New England Biolabs, Inc., Beverly, MA. IPTG and all other chemicals were obtained from Sigma Chemical Co., St. Louis, MO. A synthetic mucin peptide corresponding to the mucin core probe and the poly mucin-peptide, which was chemically polymerized with synthetic mucin peptide by reaction with either glutaraldehyde or dicyclohexylcarbodiimide, were purchased from Biosynthesis, Inc. A plasmid which contains seven mucin tandem repeats (pMuc7) (14) and the tumor-specific monoclonal antibody SM3 (11) were obtained from S. J. Gendler, Imperial Cancer Research Fund, London, United Kingdom. pMAL-p vector and expression system was obtained from QIAGEN, Inc., Studio City, CA. Cell lines used in this study were obtained from and cultured as recommended by American Type Culture Collection (Rockville, MD) MCF-7, SK-BR-3, BT-549, and BT-20 are breast tumor cell lines. Hs 578Bst is a normal breast cell line and Hep G2 is a human hepatoma cell line used as a negative control. CV-1 is an African green monkey kidney cell line. SK-BR-3, MCF-7, and BT-20 were shown to be killed by a cytotoxic T lymphocyte line that recognizes tumor-specific mucin as its target (15) and thus must express the tumor-specific epitope of mucin. BT-549 secretes a mucin-like material (American Type Culture Collection).

Construction of MBP-Muc7 Fusion Gene (16). pMuc7 was digested with EcoRI and the 5.0-kilobase fragment, which contains the seven mucin tandem repeats, was isolated by electroelution from an agarose gel. Then this fragment was cloned into an expression vector, pMAL-p, and transformed into E. coli DH5α strain. The clones that were ampicillin resistant were selected and their plasmid DNA was obtained by a rapid minilysis procedure. The DNA was analyzed by restriction endonuclease digestion and the correctly oriented recombinants were selected for expression.

Expression and Purification of MBP-Muc7 Chimeric Protein. Bacterial strain DH5α containing plasmid was grown at 37°C in M9 media to an A600 of 0.6, at which point IPTG was added to a final concentration of 0.3 mm to induce the lac promoter. Cultures were allowed to grow for an additional 2 h at 37°C. For protein analysis, cells obtained from 1.0 ml of culture were harvested by centrifugation, resuspended in 50 ml lysis buffer (10 mm Tris-HCl, 0.1 mm EDTA, 0.1 mm phenylmethyl sulfonyl fluoride), and sonicated to release the protein. After centrifugation at 9000 g for 30 min, the crude extract supernatant was loaded onto an amylose resin column and the fusion protein was then eluted with 10 mm maltose (17).
RECOMBINANT BREAST TUMOR-ASSOCIATED MUCIN

Fig. 1. Expression of MBP-mucin fusion protein in E. coli versus the time after induction with IPTG. Cells were grown in M9-CA media to late log phase at which time IPTG was added to a final concentration of 0.3 mM. After harvesting, the cells were dissolved in SDS sample buffer and subjected to 10% SDS-PAGE. A, Coomassie blue staining; B, immunoblot of same gel with MAb SM3; kd, molecular weight in thousands.

Enhanced Chemiluminescence Immunoblot Analysis. Bacteria or purified protein was dissolved in SDS-sample buffer (18), boiled 5–10 min, and electrophoresed on 8% SDS-PAGE. Then proteins were transferred to nitrocellulose membranes. Filters were blocked with 4% bovine serum albumin in 10 mM Tris (pH 7.5)-.15 mM NaCl-0.05% Tween 20 buffer, washed with this buffer and incubated with rabbit anti-rMucin at room temperature (1:3000 dilution, 40 min). Filters were then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Kirkgaard & Perry Laboratories, Inc.) (1:30,000, 30 min). After a washing the filters were transferred to a cyclic diacylhydrazides solution (Amersham, Inc.) for 1 min and exposed to X-ray film for 1–15 min.

Production of Antisera against MBP-Mucin. New Zealand White rabbits were immunized by s.c. injection at multiple sites of 200 µg of peptide or 50 µg of MBP-mucin in a 1:1 emulsion with Freund's complete adjuvant in a final volume of 1 ml. Control rabbits received either phosphate-buffered saline or 50 µg MBP in a 1:1 emulsion with Freund's complete adjuvant. Two rabbits were immunized with each emulsion. After 3 weeks, the rabbits were given a second injection of the same immunogen in a 1:1 emulsion with incomplete adjuvant. Blood samples were obtained from the marginal ear vein on days 0 and 21 and at biweekly intervals thereafter.

Cellular ELISA. Cells (10⁴) were seeded into each well of the 96-well flat-bottomed tissue culture microtiter plate and cultured overnight at 37°C. The cells were washed once with phosphate-buffered saline and fixed with 100% methanol at –20°C for 10 min. One hundred µl of rabbit antiserum with a minimum 1:100 dilution were added to each well and incubated for 1 h at room temperature. Cells were washed 3 times with phosphate-buffered saline and then incubated with goat anti-rabbit IgG(H+L) conjugated to horseradish peroxidase for 1 h at room temperature. Then the plates were washed again and incubated with 2,2' azinodio[3-ethylbenzthiazoline sulfonate] (Kirkgaard & Perry Laboratories, Inc.) as substrate and read at a wavelength of 410 nm with a microplate reader.

Immunofluorescence Staining of Breast Tumor Cell Lines. Breast tumor cells were seeded in 24-well tissue culture plates and incubated for 16 h. Cell monolayers were fixed with 100% methanol at –20°C for 10 min. Rabbit antiserum with a minimum 1:50 dilution was added to each well and incubated for 1 h at room temperature. The immune complexes were visualized by incubation with fluorescein-conjugated goat anti-rabbit IgG (Caltag Laboratories, Inc.; 1:50 dilution, 1 h, room temperature) and photographed using UV emission microscopy.

Fig. 2. SDS-PAGE of purified MBP-mucin fusion protein. Samples were fractionated on 10% SDS-PAGE and then visualized by Coomassie blue staining. Lane 1, purified MBP-mucin; lane 2, purified MBP-mucin fusion protein after treatment of protease Factor Xa overnight; lane 3, purified MBP-mucin treated with protease Factor Xa for 4 h at room temperature; lane 4, 0.5 h after treatment with protease Factor Xa; kd, molecular weight in thousands.
Dilution

Fig. 3. ELISA assays of mucin MAh SM3 and rabbit anti-rMucin with MBP-mucin (rMucin), synthetic mucin peptide (peptide), and human milk fat globulin (HMFG). A, MAh SM3; B, rabbit anti-rMucin. Bars, SD.

RESULTS

Construction, Expression, and Isolation of MBP-Mucin Chimeric Protein. The MBP-muc7 was constructed as described under "Materials and Methods." The seven tandem repeats of mucin cDNA fragment were inserted downstream of the malE gene of expression vector pMal-p, which encodes the maltose-binding protein, and resulted in expression of a MBP-mucin fusion protein. Since the MBP has high affinity to maltose, this construction provided a one-step purification by affinity chromatography using an amylose resin column. The chimeric protein was then eluted with 10 mM maltose. The fusion protein also contains the recognition site of the specific protease Factor Xa, located between the MBP and the mucin core repeats. This allowed MBP to be cleaved from the fusion protein after purification.

In order to express the MBP-muc7 fusion protein, the plasmid DNA containing the correctly oriented insert was purified and transfected into recA(-) bacterial strains HB101 and DH5α. Growth of the bacterial strains carrying the MBP-muc7 gene in the presence of 0.3 mM IPTG resulted in the production of a 65,000 protein. The maximum production was reached at 4 h after induction with IPTG (Fig. 1A). This protein was readily stained with breast tumor-specific monoclonal antibody SM3 (Fig. 1B). As shown in Fig. 1, the addition of IPTG resulted in elevated levels of chimeric protein being produced. On the other hand, a small amount of product was observed in the

Table 1 Summary of the rabbit antisera titers

<table>
<thead>
<tr>
<th>Rabbit</th>
<th>Immuneogen</th>
<th>MBP-mucn</th>
<th>Mono-muc peptide</th>
<th>Poly-muc peptide</th>
<th>MBP</th>
<th>HMFG</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mono-muc peptide</td>
<td>3.0 × 10⁴</td>
<td>2.1 × 10⁶</td>
<td>2.4 × 10⁵</td>
<td>ND</td>
<td>2.7 × 10⁵</td>
</tr>
<tr>
<td>2</td>
<td>Mono-muc peptide</td>
<td>1.0 × 10³</td>
<td>1.0 × 10⁵</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>MBP-mucn</td>
<td>6.3 × 10⁶</td>
<td>2.7 × 10⁵</td>
<td>ND</td>
<td>6.3 × 10⁶</td>
<td>1.0 × 10⁵</td>
</tr>
<tr>
<td>4</td>
<td>MBP-mucn</td>
<td>1.9 × 10⁷</td>
<td>7.2 × 10⁵</td>
<td>2.4 × 10⁵</td>
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<td>1.0 × 10⁵</td>
</tr>
<tr>
<td>5</td>
<td>Poly-muc peptide</td>
<td>ND</td>
<td>ND</td>
<td>1.0 × 10³</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>6</td>
<td>Poly-muc peptide</td>
<td>ND</td>
<td>ND</td>
<td>2.0 × 10³</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
<td>MBP</td>
<td>2.1 × 10⁶</td>
<td>ND</td>
<td>6.3 × 10⁶</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Mono-muc peptide, synthetic mucin peptide corresponding to a single mucin tandem repeat; Poly-muc peptide, chemically polymerized synthetic mucin peptide; HMFG, human milk fat globulin; ND, not detectable.
**Fig. 5. Cellular ELISA of breast tumor cell lines with rabbit antisera (1:50 dilution).** MCF-7, SK-BR-3, BT-549, and BT-20 are breast tumor cell lines. Hs 578Bst is a normal breast cell line and Hep G2 is a human hepatoma cell line used as a negative control. pep, peptide. Bars, SD.

Absence of inducer (Fig. 1, Lane m). The chimeric protein stained with rabbit anti-MBP has a pattern similar to that of MAb SM3 (data not shown).

**Purification of MBP-Mucin Hybrid Protein.** The wild-type MBP binds maltose and $\alpha$-(1-4)-glucose polymers with $K_M$ of $10^{-6}$ to $10^{-7}$ M (17). This property can be used for an easy and rapid purification by affinity chromatography on cross-linked amylose columns (19). To demonstrate that MBP-mucin retains the maltose binding activity, samples were incubated with cross-linked amylose. The amylose matrix was separated from the supernatant by centrifugation, washed, and incubated with 10 mm maltose. Supernatants analyzed by SDS-PAGE and immunoblot showed that MBP-mucin fusion protein was retained on the amylose column and eluted by maltose (data not shown). The large scale purification of MBP-mucin was performed on an amylose column as described in “Materials and Methods.” Fig. 2 shows the SDS-PAGE of the fusion protein purified from an amylose column. The major band has a molecular weight of about 65,000. Immunoblot with MAb SM3 showed that all of the bands were recognized by SM3, indicating that all of these bands contain the tandem repeats. After treatment of the fusion protein with protease Factor Xa, a new $M_c$, 42,000 band corresponding to MBP appeared (Fig. 2, Lanes 2, 3, and 4).

**ELISA Assays of Recombinant Mucin Fusion Protein with MAb SM3.** To determine whether the undernaturated recombinant mucin fusion protein has the tumor-specific epitope, an ELISA assay using purified recombinant mucin was performed. Fig. 3A shows the reactivities of a breast tumor-specific monoclonal antibody SM3 with recombinant mucin, synthetic mucin core peptide, and human milk fat globulin. MAb SM3 showed strong affinity to recombinant mucin compared to the synthetic mucin core peptide (Fig. 3A). The strong binding activity to SM3 suggests that the recombinant mucin fusion protein exposed the tumor epitope due to the unglycosylated form expressed from E. coli and resulted in increasing affinity to MAb SM3.

**Immune Response to MBP-rMucin in Rabbits.** The antibody response to the MBP-mucin fusion protein, synthetic peptide, and chemically polymerized mucin peptide was examined by ELISA. Both synthetic peptide and recombinant mucin elicited specific anti-
body response with the titers of the antisera ranging from $10^4$ to $>10^6$ (Table 1). The recombinant MBP-mucin fusion protein gave the highest immune response and its antisera reacted with both recombinant MBP-mucin and synthetic mucin peptide monomer (Table 1, rabbits 3 and 4). Chemically polymerized mucin core peptide gave a very low immune response and the antibodies recognized only the polymerized mucin peptide but not the mucin peptide monomer. Chemical polymerization of the mucin peptide may have caused the modification of side chains of mucin peptide and may have resulted in the conformational change of this peptide.

Characterization of Antibodies against MBP-rMucin. Fig. 3B shows the reactions of anti-rMucin with recombinant mucin, synthetic peptide, and human milk fat globulin. Anti-rMucin has relative high affinity to synthetic mucin peptide, while to the native human milk fat globulin the binding occurred only in the presence of high concentration of anti-rMucin. (At dilutions $\geq 1:900$, anti-rMucin is nonglycosylated and mucin specific). This may be due to its highly glycosylated form which hides the epitopes and prevents the binding of anti-rMucin raised from the unglycosylated form. To confirm this hypothesis, the competition assay of anti-rMucin with monoclonal antibody SM3 was tested and the results are shown in Fig. 4. The assays used MBP-mucin coated plates and 1:100 dilution of MAb SM3 with varied concentrations of rabbit antisera as described in “Materials and Methods.” The 82% competition of anti-rMucin indicates that at least some of the antibodies to rMucin bind the same epitope or near the epitope that MAb SM3 recognizes.

Reactivity of Rabbit Anti-rMucin with Breast Tumor Cell Lines. To test whether the rabbit antisera recognized breast tumor cell lines, a cellular ELISA assay which uses fixed cells as antigen was applied. The anti-rMucin recognized all of the breast tumor cell lines we tested (Fig. 5). The reactivities of mucin monoclonal antibodies and rabbit anti-rMucin with the same breast tumor cell lines showed similar patterns. Of the tumor cell lines, MCF-7 and SK-BR-3 gave the highest reactivity and the BT-20 and BT-549 gave relatively low or less activity. Even though Hs 578Bst, a normal breast cell line, showed reactivity to high concentrations of anti-rMucin, all tumor cell

Fig. 6. Immunofluorescence analysis of breast tumor cells with rabbit anti-rMucin. A and B, SK-BR-3 (A) and BT-20 cells (B) stained with anti-rMucin; C and D, SK-BR-3 (C) cells and BT-20 (D) cells stained with anti-MBP as controls; E, Hs 578Bst stained with anti-rMucin as a negative control.
Tumor-associated antigens have considerable promise as targets for active or passive immunotherapy. A number of studies have been directed to the identification of tumor-associated markers that could be used as a target for either active or passive immunotherapy (20, 21). Most of these studies, however, have utilized antibodies generated against undefined structures on the malignant cell surface. A more effective strategy may be to direct active immunization against a defined tumor cell surface molecule which contains a tumor-specific epitope. In this study, the intention was to use the recombinant unglycosylated mucin as a model for exposure of tumor-associated epitopes and to evaluate the potential of diagnosis, immunotherapy, and vaccine development.

Recent studies have indicated that the carbohydrate structure of mucin from malignant breast cells is different from normal cells (10, 22). These findings suggested that differential glycosylation of the protein core may result in exposure of a new epitope. Indeed, several research groups have generated MAbs that selectively reacted with transformed cells by using deglycosylated mucin (11–13). Therefore, it is reasonable to predict that the unglycosylated rMucin may contain the tumor-specific epitope and may be useful in immunotherapy or as a breast tumor vaccine.

The recombinant hybrid mucin has very high affinity to MAb SM3 compared to the synthetic mucin peptide or polymerized synthetic peptide, although all of these three contain the same 20-amino acid sequences without any carbohydrate. This indicates that the binding affinity of MAb SM3, which binds to residues Pro→Asp→Thr→Arg→Pro of mucin tandem repeats (23), may not be contributed only by these amino acids. The whole environment of surrounding amino acids or repeats may influence the conformation of a tumor epitope even where no carbohydrate occurs. The fact that the antibodies against rMucin recognize tumor cell lines in both ELISA and immunoblot, while the antibodies against synthetic mucin peptide do not, suggests that the recombinant mucin fusion protein expressed in E. coli may have potential value in both immunotherapy and/or vaccine development for breast carcinoma.

Fig. 7. Immunoblot of tumor cell lines with anti-rMucin. The tumor cells (10⁴ each) were dissolved in SDS sample buffer, fractionated on 10% SDS-PAGE, transferred to nitrocellulose membranes, incubated with anti-rMucin at 1:5000 dilution, and reported by peroxidase conjugated goat anti-rabbit IgG using chemiluminescence methods as described in “Materials and Methods.” BT-20 and MCF-7, breast tumor cell lines; Hep G2 and African green monkey kidney epithelial cell line CV-1, non-breast cell lines as negative controls.

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REFERENCES


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