Reversible Disruption of Cell-Matrix and Cell-Cell Interactions by Overexpression of Sialomucin Complex

(Received for publication, September 29, 1997, and in revised form, October 8, 1997)

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Sialomucin complex (SMC) is a large, heterodimeric glycoprotein complex composed of mucin (ASGP-1) and transmembrane (ASGP-2) subunits and expressed abundantly on the cell surface of ascites 13762 rat mammary adenocarcinoma cells. We have isolated recombinant cDNAs containing different numbers of ASGP-1 mucin repeats, which can be expressed as protein products with variable lengths. To study the anti-adhesive effect of SMC, these cDNAs were transfected into human cancer cell lines. Using a tetracycline-responsive, inducible expression system, we demonstrated that the overexpression of SMC induces morphology changes, cell detachment, and cell-cell dissociation of transfected A375 human melanoma cells in culture. The transition between the adherent and suspension states of the cells is fully reversible and dependent on the SMC expression level. The anti-adhesion effect of SMC was further analyzed kinetically by measuring the cell adhesion of transfected A375 melanoma and MCF-7 breast cancer cell lines to fibronectin, laminin, and collagen IV, demonstrating that SMC disrupts integrin-mediated cell adhesion to extracellular matrix proteins. The degree of this anti-adhesion effect was dependent on the number of mucin repeats in the SMC molecule as well as the level of cell surface expression.

In malignant cancer formation cells in the primary neoplasm undergo phenotypic changes and progress to invasive and metastatic stages (1). Tumor cells metastasize by a multistep process as follows: proliferation and invasion of malignant cells at primary sites; penetration into the lymphatics, blood circulation, and body cavities; transport; extravasation at distant primary sites; penetration into the lymphatics, blood circulation, and body cavities; transport; extravasation at distant tissue or organs; and invasion and growth at the secondary site (1). The tumor cells must also evade immune destruction during the metastatic process. Many of these processes involve aberrant properties of tumor cell surfaces (2), which are acquired through altered expression of cellular genes. One of the critical steps in the metastasis process is detachment of tumor cells from the primary neoplasm. Molecular mechanisms of this process are poorly understood (2). For tumor cells to be released from the primary site, tumor cell adhesions to neighboring cells and the basement membrane must be partially or completely abolished. Decreased levels of integrins and/or E-cadherin are found in many invasive and metastatic cancer cells, suggesting that the poor adhesiveness of the cancer cells may be a prerequisite for malignant cancer with a high metastatic potential (3, 4). However, other metastatic cancer cells retain their normal levels of adhesion molecules (3). One explanation for these observations is that the surface of these metastatic cancer cells is covered by anti-adhesive molecules, such as membrane mucins (5, 6), which mask cell adhesion molecules.

SMC$ is a cell surface mucin abundantly expressed by highly proliferative and metastatic 13762 rat mammary adenocarcinoma ascites sublines (5). It is distinct in size and composition from other well known membrane mucins, such as MUC1 and leukosialin (CD43) (7, 8). SMC is a heterodimeric glycoprotein complex composed of a large mucin subunit ASGP-1 and a transmembrane subunit ASGP-2 (9). The complex is synthesized as a precursor polypeptide, encoded by a single gene (10, 11), and cleaved into its two subunits at an early stage of its biosynthesis (12). The mucin subunit ASGP-1 contains 11 full repeats of $\sim 125$ amino acid residues each plus two partial repeats (11) and is heavily O-glycosylated on serine and threonine residues (13, 14). Previous studies suggest that the SMC may be involved in a protective function, diminishing cancer cell killing mediated by NK cells, macrophages, and cytotoxic T-cells (5, 15, 16). In normal epithelial tissues membrane-associated mucins are expressed only on the apical surface of the cell (5, 17). In contrast, mucins are often overexpressed in nonpolarized tumor cells, raising the question whether mucins dispersed over the cell surface facilitate tumor metastasis by altering the adhesion properties of the cells. Studies of the modulation of cell adhesion by mucins such as epiligrin and epiligrin (MUC1) suggest that these mucins prevent integrin-mediated cell-matrix and E-cadherin-mediated cell-cell interactions (18–20).

Previously, we showed by immunological analyses of human breast cancer cells in solid tumors and tumor effusions that the more aggressive cells in effusions tend to express SMC, whereas cells within the solid tumors did not (21), suggesting a role for SMC in tumor progression. Furthermore, we have observed more than a 100-fold decrease in the expression of SMC in cells of an ascites 13762 subline after selection for growth in culture (22). We propose that overexpression of SMC causes tumor cell release from a primary site by its potent anti-adhesion effect which ultimately leads to metastasis. We

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* This research was supported in part by Grant CA 52498 from the National Institutes of Health, a grant from Amgen, Inc., and by Grant CA 14395 from the Sylvester Comprehensive Cancer Center. 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: SMC, sialomucin complex containing ASGP-1 and ASGP-2; ASGP, ascites sialoglycoprotein; RT-PCR, reverse transcription-polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; PBS, Dulbecco’s phosphate-buffered saline without calcium; mAb, monoclonal antibody; BSA, bovine serum albumin; ECM, extracellular matrix; kbp, kilobase pair(s).
have therefore tested the anti-adhesive effects of SMC in vitro by transfecting the SMC gene into human cancer cell lines. For this purpose we have generated recombinant cDNAs for SMC which vary in the number of mucin repeats. These cDNAs were transfected and expressed under the control of tetracycline-responsive inducible promoter. Here we show that the human melanoma cell line A375 cells undergo a distinct morphological change and lose cell adhesion to plastic and to neighboring cells in culture when SMC expression is induced to the high levels that are found in 13762 ascites cells. Most importantly, the tetracycline-regulated expression enabled us to vary the cell surface level of SMC and to demonstrate a reversible transition between the adhesive and nonadhesive states of cells which is controlled by SMC level.

MATERIALS AND METHODS

Generation of cDNA Constructs and Transfection—The oligonucleotide W8 (GATCTGACCGGTATC) was used to prime the reverse transcription reaction to synthesize the first strand cDNA encoding the 5’-half of the entire cDNA sequence. cDNA was then amplified by PCR between L33 (L33 (TTCTCTGCTGGAGATTCCTCAGGGAGCCT-TAG) and R33 (TTAGATGTACCGGTAGTACTTGCTGGCGGT) primers (Fig. IA). The SMC-specific oligonucleotide primers used for the hybridization were T5 (TGTCGTTGTTGGTTAGATGCTC), A1-S (TTCTCTGAGCCTGAGGAGC), and A1-R (CTCTCTGGAAGAATGATTG). The amplifiers obtained were ligated with the 3’-5’-kb fragment at a unique DraIII site to generate cDNAs for the functional SMC analogs. These constructs were then subcloned into mammalian expression vectors. The inducible expression vector puHD10-3 and the transactivator plasmid tTA for expressing transactivator gene were kindly provided by Dr. Kathleen Kelly (National Institutes of Health). pcDNA3 vector was purchased from Invitrogen (San Diego).

A375 human melanoma cells and MCF-7 human breast cancer cells were grown in the complete media recommended by the American Type Culture Collection. These cell lines were transfected with cDNAs for SMC analogs and tTA using LipofectAMINE reagent (Life Technologies, Inc.). Cells were then cultured in the G418- and hygromycin-containing media for the selection of the transfectants. Drug concentrations for the selection were 0.8 mg/ml G418 and 0.3 mg/ml hygromycin for A375 cells and 0.5 mg/ml G418 and 0.07 mg/ml hygromycin for MCF-7 cells. Clones with high expression levels of SMC-variants were selected from the drug-resistant populations by fluorescence-activated cell sorting following the cell surface staining with the anti-ASGP-2 mAb 4F12 and fluorescein isothiocyanate-conjugated secondary antibodies (Sigma).

Antibodies—Monoclonal antibodies against ASGP-2 (4F12 and 13C4) were purchased previously (22). Anti-ASGP-1 polyclonal antiserum was raised against a synthetic peptide contained in the sequence near the C-terminus of human ASGP-1, and reacts with the ASGP-1 from rat mammary adenocarcinoma 13762 cells.

FACScan Analysis—Cells from subconfluent cultures were detached gently from the plate by treating with PBS- and EDTA-containing dissociation buffer (Life Technologies, Inc.). Cells were washed 2–3 times with PBS and resuspended in PBS containing 3% BSA. Subsequently, cell suspensions were incubated with anti-ASGP-2 mAb 4F12 or anti-integrin α5 mAb (Life Technologies, Inc.), followed by incubation with fluorescein isothiocyanate-conjugated secondary antibodies (Sigma). The fluorescence intensity was determined by FACScan analysis. The number of SMC molecules per cell was estimated by comparison with 13762 ascites cells, whose SMC content was determined from their sialic acid analyses (13).

Cell Aggregation and Adhesion Assays—Cells were detached from the plate by treating with dissociation buffer as described above. After rinsing three times with PBS, cells were resuspended in the adhesion assay media (Dulbecco’s modified Eagle’s medium, 0.25% BSA). The viability of the cells was checked by trypsin blue exclusion. At least 95% viability was confirmed before each assay. Cells suspensions (1 × 106 cells in 1.5 ml) were added to each ECM-coated well and incubated at 37 °C for the different times. After removal of the non-adherent cells by washing the wells twice with PBS, the remaining adherent cells were lysed with 0.8% Triton X-100 in PBS. The relative cell number was determined from aliquots of cell lysates using the CytoTox 96 assay kit (Promega), as described by the company. The number of adherent cells was determined as the percentage of the total number of cells added to each well. For correction of the nonspecific binding of cells, the number of cells bound to the BSA-coated wells was subtracted from the value of the experimental wells. Each experiment was performed in duplicate.

RESULTS

Construction of SMC Expression Plasmids—The 9-kbp sequence of the sialomucin complex precursor (pSMC-1) mRNA was determined from several overlapping cDNA clones which were obtained by several different cloning methods, including 5’-rapid amplification of cDNA ends technology (10, 11). A single, contiguous cDNA containing the complete pSMC-1 coding region has never been isolated. To perform functional analyses of the sialomucin complex by transfection and expression in different mammalian cell lines, it was necessary to generate pSMC-1 cDNA in an appropriate expression vector. The largest single cDNA previously obtained is the 3’ 5-kbp of the pSMC-1 message. This cDNA contains the entire ASGP-2 coding region and 544 bases of the ASGP-1 3’ sequence, beginning in the repeat region (Fig. IA). The strategy for constructing the full-length cDNA required generating the remaining 5’ end of the pSMC-1 mRNA by RT-PCR and ligating this amplification product to the 3’ 5-kbp cDNA sequence. For this purpose, first strand cDNA was synthesized by reverse transcription using poly(A) RNA from 13762 MAT C-1 ascites cells as a template and W8, which is contained near the 5’ end of the 3’ 5-kbp cDNA clone and is 3’ of a unique restriction endonuclease site DraIII, as a primer. This site could be used for the subsequent ligation of the 5’ and 3’ cDNAs (Fig. IA). The 5’ cDNA was then amplified by PCR between 5’ primer L33 from the 5’-untranslated region and 3’ primer R33 which is nested to the W8 primer (Fig. IA). The expected PCR product is about 5 kbp in size and contains part of the 5’-untranslated region, the translation initiation codon, all of the tandem repeat sequence, and a part of the ASGP-1 3’-unique sequence. Following RT-PCR, amplification products were separated by agarose gel electrophoresis, blotted to nitrocellulose, and further identified by hybridization with an additional oligonucleotide, T5, which is contained in the muin repeat sequence of ASGP-1. Surprisingly, many bands were observed, ranging from the size of ~400 base pairs to ~6 kbp (data not shown). To investigate the origin of these products, they were cloned into pCRII vector (Invitrogen) and screened for the presence of ASGP-1 sequences by hybridization with two additional gene-specific oligonucleotide probes: A1-5’ and an oligonucleotide probes: A1-5’, whose sequence spans the translation initiation site of pSMC-1, and A1-R, a sequence from the 3’-unique region of ASGP-1 (Fig. IA). Positive hybridization to these oligonucleotides indicated that the inserts of these plasmids were authentic amplification products of the pSMC-1 message, since they included these nested sequences from both the 5’ and 3’ ends. The sizes of these inserts were determined by digesting the plasmid DNA with EcoRI and separating the fragments by agarose gel electrophoresis and found to vary between 500 base pairs and 4 kbp, corresponding to the products observed from the original RT-PCR (Fig. IB).

Further confirmation that these indeed cDNA sequence encoding ASGP-1 was obtained by DNA sequence analysis. The nucleotide sequences of the 3’ and 5’ ends of selected clones, clone R15 (3.8 kbp), clone h (2.4 kbp), clone e
As expected, sequencing indicated that these clones were identical to ASGP-1 and contained the 5'- and 3'-unique sequences and the repeat region (Fig. 2A). These observations lead to the interpretation that differences in the sizes of the RT-PCR products result from the fact that each amplimer contains a different number of the tandem repeats. Sequencing within the repeat domains is difficult because of annealing of the primers at multiple sites in the repeat sequences. Thus, it was possible to perform complete sequencing of only the two smallest clones, clone f and clone e. The number of mucin repeats in each clone was estimated by the sizes of the insert. Clone R15, clone h, clone e, and clone f contain approximately 8, 5, 3, and 1 repeats, respectively. Amino acid sequence deduced from the DNA sequence showed a high content of serine (20%), threonine (31%), and proline (6%) residues in the repeat sequence, as expected for mucin-type glycoproteins (Fig. 2B). These observations provided evidence that we have generated recombinant cDNAs for ASGP-1 by RT-PCR which are different only in the number of mucin repeats.

The mechanism for the generation of these multiple amplimers undoubtedly resides in the high degree of homology in the tandem repeat sequences (Fig. 3). During the polymerase chain reaction, especially with long PCR times, DNA polymerase often terminates before completing the replication of the template strand. In the case of pSMC the incomplete copy often ended within the repeated sequence domain. These incomplete strands accumulate during the cycles and can undergo annealing, due to the high homology within the repeat sequences. The polymerase would then act as a primer from the ends of these partial sequences and fill in the remaining portion to complete the synthesis of a shortened, double-stranded ASGP-1 sequence, creating a deletion within the repeat domain. These shorter ASGP-1 sequences would then be amplified in the later PCR cycles. Therefore, recombinant cDNAs for ASGP-1 with the different number of repeats have been produced. These derivatives permit us to investigate the role of mucin size and repeat number in mucin function.

Each of the different ASGP-1 cDNAs was ligated to the 5-kbp 3' clone at the DraIII site to yield ASGP-1/ASGP-2 cDNA constructs, rep8, rep5, rep3, and rep1. Relative sizes of these recombinant cDNAs were compared with the native form (Fig. 4). To express SMC in mammalian cell lines under the control of a tetracycline-responsive promoter, constructs rep8, rep5, and rep3 were subcloned into the inducible expression vector pUHD10-3. Construct rep1 was subcloned into pcDNA3 (Invitrogen) for constitutive expression.

Expression of SMCs with Varying ASGP-1 Lengths in Human Cancer Cell Lines—Contributions of sialomucin complex to cell surface properties can be best studied by transfecting the SMC gene into cell lines and altering the expression level using an inducible system. We transfected SMC cDNA constructs into A375 human melanoma and MCF-7 human breast cancer cell lines to determine whether SMC cell surface expression alters the adhesive properties of these cells. Two cell lines were chosen because neither contains endogenous SMC at a detectable level. The tetracycline-responsive expression system has been established for highly efficient regulation of transfected genes in mammalian cells (23). In this system tTA protein, a fusion protein of the tet repressor of Escherichia coli and the activating domain of herpes simplex viral protein VP16, binds to tet operator sequences from Tn10 (tetO) fused to a...
minimal promoter sequence and activates transcription of the gene inserted downstream of the tetO promoter. The tetO promoter is virtually silent without activation by tTA. Since tTA does not bind to the tetO sequence in the presence of the antibiotic tetracycline, transcriptional activation by tTA can be repressed by addition of tetracycline to the culture media.

For the regulation of the expression of SMC, cDNAs for SMC in tetO vector (PCH10-3) and transactivator (tTA) gene were co-transfected into the cell lines. During selection of inducible transfectants, cells were grown in tetracycline-containing media (2 μg/ml) to repress expression of SMC. Expression of SMC was induced in transfectants by removing tetracycline from the culture media, and protein products were characterized by SDS-PAGE/immunoblot analysis with anti-ASGP-2 mAb 13C4. Transfectants of both A375 and MCF-7 cells expressed ASGP-2 with a size similar to ASGP-2 in 13762 ascites cells (Fig. 5A).

The sizes of ASGP-2 protein expressed from all four different cDNAs, Rep8, Rep5, Rep3, and Rep1, were identical in each cell type. These data indicate that precursors of the recombinant proteins are processed in the same manner as the native sialomucin complex in ascites cells by proteolytic cleavage of the precursor into two subunits and co-/post-translational glycosylation (12). This observation was confirmed by a pulse-chase analysis in which mature ASGP-2 first appears in a 30-min chase (Fig. 5B). A375 cells expressing Rep1 SMC analog were metabolically labeled with [35S]methionine and cysteine for 10 min and chased for the indicated times. Cells were then lysed in 0.5% SDS lysis buffer, and after the addition of Nonidet P-40 to the cell lysate, the precursor protein and mature ASGP-2 were immunoprecipitated using anti-ASGP-2 polyclonal antibodies. Following SDS-PAGE, proteins were detected by fluorography. In this pulse-chase experiment, the ASGP-1 molecule disappears after the cleavage of the precursor protein pSMC, because association of the two subunits is disrupted by...
When inducible transfectants were cultured with the constitutive expression of Rep1 were also expressed at a similar level. When inducible transfectants were cultured with the constitutive expression of Rep1 were also expressed at a nearly 100-fold increase in the cell surface level of SMC. When induced, SMC was strongly expressed on the cell surface. To answer this question in vitro, we varied the cell surface levels of SMC in A375 cell lines using the inducible expression system. At low levels of cell surface expression (~10,000 molecules/cell), SMC did not show a significant effect on the morphology of these cells compared with the parent line. When overexpression (~10⁶ molecules/cell) was induced, however, SMC demonstrated a potent anti-adhesive effect. In the absence of tetracycline in the culture media, A375 clone Rep8, expressing the eight-repeat mucin, completely altered its cell morphology to a spherical shape and became detached from the tissue culture dish (Fig. 8, A and B). Cells remained in suspension and did not aggregate even at high density, indicating that overexpression of SMC on the cell surface strongly disrupts both cell-cell and cell-matrix adhesion. Moreover, reduction of SMC level ~100-fold by addition of tetracycline to the culture media restored cell adhesion within 48 h (Fig. 8, B and C). The transition between the suspension and adherent states of the cells was fully reversible and regulated by SMC level (Fig. 8, A–D). Control experiments indicated that tetracycline had no effect on the adhesion of the parental cell line.

Disruption of cell-cell interactions by SMC was further demonstrated in an aggregation analysis in which cells were seeded at very high density (3 × 10⁶ cells/24 well plate) in complete media and incubated for 24 h at 37 °C. Cells were seeded on agarose-coated plates to prevent cell attachment to the plates. In the presence of tetracycline in the media, cells formed large aggregates, suggesting the association of cell adhesion molecules on adjacent cells (Fig. 9A). In the absence of tetracycline, however, no cell-cell adhesion was observed after the prolonged incubation (24 h) (Fig. 9B). Cells remained as single cells in suspension, although neighboring cells clearly made contact with each other at the density tested. These non-aggregating cells were divided into two wells, and tetracycline was added to one of them to repress SMC expression. In the control well, in which cells were incubated without tetracycline, cells still remained as single cells without aggregation (Fig. 9C). In contrast, the cells incubated with tetracycline formed aggregates.

SDS lysis. Next, we compared the molecular weight of ASGP-1 expressed from the different cDNA constructs. Each transfectant was lysed in the Nonidet P-40 lysis buffer, and SMC was immunoprecipitated using anti-ASGP-2 mAb 13C4. Immunoprecipitates were analyzed for the presence of ASGP-1 and its molecular weight by immunoblotting with anti-ASGP-1 polyclonal antibody HA-1 following SDS-PAGE. ASGP-1 was detected in each transfectant, with the apparent M₆ varying between 170 and ~300. The apparent correspondence in migration of the 5 and 8 repeat forms is not too surprising, since migration of large, highly glycosylated proteins on SDS-PAGE is often anomalous, because it is dependent on charge as well as size. Overall, these results have demonstrated that the two subunits, ASGP-1 and ASGP-2, are associated and present as a complex in these transfected cell lines, as they are in 13762 ascites cells, and that the size of ASGP-1 differs depending on the size of the transfected cDNA construct (Fig. 5C). By CsCl density gradient centrifugation, we also demonstrated that the densities of SMC molecules increase with the increasing number of mucin repeats, indicating an increased amount glycosylation on the SMC proteins (data not shown). These results show that the expressed SMC analogs have the properties expected and required for the study of SMC function.

Induction of SMC expression and analyses of the expression levels on the cell surfaces in the transfectants were monitored by FACSscan analysis. When induced, SMC was strongly expressed on the cell surface of A375 transfectants, and levels of expression were comparable to those found on the 13762 ascites cells (~10⁶ molecules/cell) (Fig. 6A). A375 transfectants with the constitutive expression of Rep1 were also expressed at a similar level. When inducible transfectants were cultured with 1–2 μg/ml tetracycline, lower levels of SMC (approximately 10,000 molecules/cell) were expressed, indicating that the expression of transfected genes was not completely abrogated by tetracycline. Nevertheless, the induction of expression resulted in a nearly 100-fold increase in the cell surface level of SMC. For cell adhesion assays comparing the cells expressing different levels of SMC, cell surface levels of integrin α5 were determined in the presence and absence of tetracycline. FACSscan analysis using anti-human integrin α5 mAb showed that removal of tetracycline and induction of SMC expression have no effect on the cell surface levels of this adhesion molecule, and the levels are similar to those on parental cells (Fig. 6B). Similar results were obtained from FACSscan analyses for MCF-7 transfectants. Both A375 and MCF-7 transfectants are therefore useful for analyses of anti-adhesive effects of SMC.

SMC Overexpression Causes Cell Detachment of A375 Human Melanoma Cells—If cell surface SMC suppresses cell-cell and cell-matrix interactions, it should affect cell morphology, adhesiveness, and motility. For malignant tumor cells with abundant SMC, inhibition of cell adhesion might be potent enough to cause tumor cell detachment from solid tumor mass, allowing tumor cells to circulate in body fluid without attaching to neighboring tissues. Supporting this idea, previous analyses in human breast cancers showed that a higher fraction of tumor cells from effusions expressed SMC than cells within solid tumors (21). We therefore compared the cell surface levels of SMC of 13762 adenocarcinoma ascites MAT-B1 cells growing in the peritoneal fluid of the rat with those of its adherent derivative subline selected to grow on plastic dishes. FACSscan analysis showed asceses cells expressed over 10⁶ molecules of SMC on the cell surface, whereas adherent sublines showed >100-fold decreased expression (Fig. 7). These findings raised a question whether the transition between adherent and non-adherent state of metastasizing tumor cells can be modulated by cell surface level of SMC. To answer this question in vitro, we varied the cell surface levels of SMC in A375 cell lines using the inducible expression system. At low levels of cell surface expression (~10,000 molecules/cell), SMC did not show a significant effect on the morphology of these cells compared with the parent line. When overexpression (~10⁶ molecules/cell) was induced, however, SMC demonstrated a potent anti-adhesive effect. In the absence of tetracycline in the culture media, A375 clone Rep8, expressing the eight-repeat mucin, completely altered its cell morphology to a spherical shape and became detached from the tissue culture dish (Fig. 8, A and B). Cells remained in suspension and did not aggregate even at high density, indicating that overexpression of SMC on the cell surface strongly disrupts both cell-cell and cell-matrix adhesion. Moreover, reduction of SMC level ~100-fold by addition of tetracycline to the culture media restored cell adhesion within 48 h (Fig. 8, B and C). The transition between the suspension and adherent states of the cells was fully reversible and regulated by SMC level (Fig. 8, A–D). Control experiments indicated that tetracycline had no effect on the adhesion of the parental cell line.

Disruption of cell-cell interactions by SMC was further demonstrated in an aggregation analysis in which cells were seeded at very high density (3 × 10⁶ cells/24 well plate) in complete media and incubated for 24 h at 37 °C. Cells were seeded on agarose-coated plates to prevent cell attachment to the plates. In the presence of tetracycline in the media, cells formed large aggregates, suggesting the association of cell adhesion molecules on adjacent cells (Fig. 9A). In the absence of tetracycline, however, no cell-cell adhesion was observed after the prolonged incubation (24 h) (Fig. 9B). Cells remained as single cells in suspension, although neighboring cells clearly made contact with each other at the density tested. These non-aggregating cells were divided into two wells, and tetracycline was added to one of them to repress SMC expression. In the control well, in which cells were incubated without tetracycline, cells still remained as single cells without aggregation (Fig. 9C). In contrast, the cells incubated with tetracycline formed aggregates.

**FIG. 3.** Model for production of the incomplete ASGP-1 segments. A, incomplete strands, resulting from dissociation of DNA polymerase from template before the completion of replication. B, these incomplete strands anneal to each other and DNA polymerase fills in to make a short double-stranded DNA. C, the subsequent PCR steps amplify the shortened ASGP-1 sequence.
within 48 h, indicating that the reduction of SMC level permits cell-cell interaction (Fig. 9D). Since small amounts of tetracycline should not alter the level or activity of adhesion molecules, and suspension/adherent states of cells were reversibly regulated by SMC expression, we conclude that SMC level is the determinant of cell adhesiveness in this in vitro model. These observations, along with the FACScan analyses of cell surface SMC levels, indicate that 2–3 × 10^5 molecules of SMC per cell are sufficient to suppress attachment of A375 cells.

**SMC Disrupts Integrin-mediated Cell Adhesion**—To understand the mechanism by which SMC disrupts cell-cell and cell-matrix adhesions, we examined quantitatively the abilities of SMC-transfected cell lines to adhere to ECM components at different levels of SMC expression. This was done by measuring adhesion of A375Rep8 (A375 cells expressing the 8-repeat mucin) and MCF-7Rep5 (MCF-7 cells expressing the 5-repeat mucin) clones to fibronectin, laminin, and collagen IV at three different time points, 5, 15, and 30 min. Adhesion assays showed that overexpression of cell surface SMC (>10^6 molecules/cell) reduced adhesion of both A375 and MCF-7 cells to each ECM component (Fig. 10). When the SMC level was at ~10,000 molecules per cell, levels of cell adhesion were intermediate compared with the adhesion of SMC-overexpressing cells and parental cells. These data demonstrated an inverse correlation between the cell surface level of SMC and cell adhesion to ECM components, indicating that cell surface expression level is a determinant of the anti-adhesiveness of SMC. Furthermore, overexpression of SMC disrupted cell adhesions to all three ECM components we tested. However, cell adhesions to the different ECM components are mediated by the different subsets of integrin subunits. Since SMC can block cell-cell adhesions as well as cell-matrix adhesions to different substrates, these combined results indicate that the inhibition of cell-cell and cell-matrix interactions by SMC is mediated by a nonspecific mechanism.

**Anti-adhesion Effect of SMC Depends on the Number of Mucin Repeats**—As suggested above, the most probable mechanism of SMC-mediated inhibition of adhesion is nonspecific steric hindrance due to the structural properties of SMC. If so, the size of the mucin subunit should be a critical factor for SMC-mediated anti-adhesive effects. Therefore, we determined whether changing the number of mucin repeats influences the

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**FIG. 4.** Size comparisons of the various cDNA constructs and the native form, pSMC-1. All cDNAs are identical in the domain structure but different in the length of the mucin repeat sequence.

**FIG. 5.** Characterization of protein products. A, SMC analogs expressed in A375 and MCF-7 cell lines were analyzed by anti-ASGP-2 immunoblotting with 13C4 mAb following SDS-PAGE. Native SMC from 13762 ascites cells was used as a reference. B, SMC analogs were immunoprecipitated from cell lysates using 13C4 mAb, and the immune complex was subjected to the immunoblot analysis with anti-ASGP-1 polyclonal antiserum HA-1. C, biosynthesis of SMC analog Rep1 was analyzed by pulse-chase analysis. A375 cells transfected with rep1 cDNA clone were metabolically labeled with [35S]methionine and cysteine for 10 min. Cultures were chased in the absence of label for the indicated times, and cells were lysed for immunoprecipitation with anti-ASGP-2 polyclonal antibodies. The precursor SMC and mature ASGP-2 were detected by SDS-PAGE and fluorography.
degree of the anti-adhesive effects of SMC. For this purpose, we compared the kinetics of cell adhesion onto fibronectin between A375 parental cells and the transfectants expressing eight-repeat (Rep8), three-repeat (Rep3), and one-repeat mucins (Rep1). For meaningful comparisons, it was shown that the three transfectants express SMC analogs on the cell surface at similar levels (Fig. 6A). Data from the adhesion assay indicated that increasing the number of mucin repeats resulted in decreasing levels of cell adhesion to fibronectin (Fig. 11), indicating that the size of the expressed mucin determines the degree of the anti-adhesive effect. This observation provided further evidence for the suggested mechanism of SMC-mediated inhibition of cell adhesions. Recombinant mucin molecules tested here are different only in the number of mucin repeats but identical in the rest of the molecule. Therefore, the size-dependent differential effects rule out the possibility that SMC inhibits cell adhesion by remodeling the cytoskeletal network or by down-regulating the activities of integrins via signal transduc-

Fig. 6. Induction of SMC expression and analysis of cell surface levels. A, the expression levels of SMC analogs in stably-transfected A375 cells were determined by FACScan analysis. 1) cell surface expression level of Rep8 SMC analog was determined before (OFF, dotted lines) and after the gene induction (ON, solid lines). The negative control (Cont., secondary antibody only) is represented by the dashed line. 2) cell surface expression level was similarly determined for Rep3. 3) Rep8 expression was determined after repression by the addition of tetracycline. 4) parental A375 cells (dashed line) and transfectants were analyzed for the constitutive expression of Rep1 SMC analog (solid line). B, the cell surface expression level of integrin α5 in each cell type with or without tetracycline in the culture media is represented by the solid line; the dashed lines represent the negative control.
tion events. Likewise, it is highly unlikely that the anti-adhesive effect of SMC is mediated by directly or indirectly interacting with integrin molecules, thereby inhibiting their binding to ECM components. Rather, our data strongly suggest full-length SMC exerts a potent anti-adhesive effect by masking the cell surface adhesion molecules with its extended and rigid structure.

**DISCUSSION**

SMC is highly expressed and globally distributed over the cell surface of the highly metastatic 13762 rat mammary adenocarcinoma ascites cells. Furthermore, selection of 13762 cells for increased metastasis demonstrated a correlation between levels of ASGP-1, called gp580, and metastasis of selected sublines (24). In this report we used a tetracycline-inducible expression system to show that up-regulation of cell surface SMC expression increasingly disrupts both cell-cell and cell-matrix interactions. The degree of this anti-adhesive effect was dependent on the size of the SMC molecule as well as the level of cell surface expression.

O-Glycosylation of mucin-type glycoproteins prevents their folding into a globular structure (25). In addition, the high proline content predicts that the mucin-type polypeptide forms a polyproline β-turn helix (26). Consequently, mucins in general have an extended rod-like structure. It is postulated from electron microscopic studies of episialin and leukosialin that the mucin domains of these glycoproteins span approximately 4–5 nm per 20 amino acids (25, 27, 28). Assuming that the whole 2172 amino acid sequence of ASGP-1 comprises a mucin-like structure, the length of this molecule can be estimated to be approximately 500 nm. Episialin (MUC1) and epiglycanin have a similar length of 450 nm, whereas other surface proteins, such as adhesion molecules, do not extend further than 30 nm above the cell surface (29, 30). Consequently, adhesion molecules expressed on the cell surface are sterically blocked by such membrane mucins and cannot reach their ligands in the extracellular matrix and on neighboring cells. The size of the extracellular domain of episialin varies between 1000 and 2200 amino acids as a result of genetic polymorphism. The extracellular domain of SMC predicted from cDNA sequence is up to 2900 amino acids long. Therefore, it is likely that sialomucin complex (ASGP-1-ASGP-2) exerts anti-adhesive effects by a similar
mechanism to that of episialin and epiglycanin. Because of its considerably larger size (Fig. 12), the effects of SMC should be more potent. The extensive glycosylation adds yet other contributions to structural properties of SMC. The carbohydrate side chains added onto the SMC protein backbone would make the molecule not only very rigid, because of the steric hindrance between the carbohydrate cores, but also very bulky. The extracellular region of the transmembrane subunit, ASGP-2 is 700 amino acid residues long and abundantly N-glycosylated. Because of the glycosylation and its transmembrane domain,

**FIG. 10. Disruption of cell-ECM interactions by SMC expression.** A375 transfectants expressing Rep8 analog and MCF-7 transfectants expressing Rep5 analog were examined along with the parental lines for the adhesion to fibronectin, laminin, and collagen IV. Each graph shows the percentage of cells bound to each ECM component in the indicated time. The adhesion of parent A375 cells to laminin and collagen IV was not determined.

**FIG. 11. Determination of the degree of the anti-adhesive effect by the number of mucin repeats.** A375 transfectants overexpressing SMC analogs containing the different number of repeats, Rep8, Rep3, and Rep1, were compared for the adhesion to fibronectin. FACScan analysis showed that all three analogs are expressed at the similar levels, as described above.

**FIG. 12. Schematic representation of the cell surface molecules.** The lengths of the extracellular portions of the cell surface molecules are as indicated and drawn to scale. The mucin subunit of SMC and SMC analogs are represented as unfilled bars. The extracellular domain of ASGP-2 is represented as a filled oval (not to scale).

this molecule is also suggested to have non-globular structure. As a result the abundantly expressed SMC masks the entire cell surface and sterically hinders adhesion molecules from binding to their substrates. Suppression of cell-cell and cell-matrix interactions by episialin/MUC1 was previously demonstrated in the transfected A375 melanoma cell line (18, 20).
From the anti-adhesive behavior observed with other membrane-bound mucins such as epiglycanin (19) and leukosialin (28, 31, 32), it can be postulated that the anti-adhesive effects are nonspecific and due to masking by their rigid and extended structures. This postulated mechanism of anti-adhesive action is supported by our observations of the suppression of cell adhesion by SMC, which is dependent on the expression level and length of the mucin molecules. It was clearly demonstrated in our study that expression level and the number of mucin repeats are critical factors for anti-adhesive property of SMC. However, the increasing number of mucin repeats not only adds to the length of the molecule but provides more potential O-glycosylation sites for addition of more negatively charged sialic acid residues. However, neuraminidase treatments of episialin to remove sialic acid residues did not abrogate its anti-adhesive effects (27), indicating sialylation is not necessary for the mucin anti-adhesive effect. Nevertheless, this result does not suggest O-glycosylation is dispensable for the function of SMC as an anti-adhesive agent, because the extensive glycosylation is presumably essential to produce the extended structure and non-flexible nature of the mucin molecule.

The importance of the anti-adhesivity of SMC is that cancer cell adhesion can be substantially reduced or completely abolished without variable expression or function of adhesion molecules such as E-cadherin and integrins. This phenomenon may provide an explanation for highly metastatic cancer cells retaining normal levels of adhesion molecules as reported previously (3). Since it is suggested that cells with altered cell-cell and cell-matrix interactions are predisposed to metastasis, our present study certainly implicates SMC in tumor metastasis. In A375 human melanoma cells SMC overexpression caused cell detachment from the culture dish and neighboring cells. Because metastatic tumor cells require successive cell release and adhesion mechanisms, anti-adhesive effects on these cells must be reversible. Importantly, adhesion of these SMC-transfected cells was restored by reducing the SMC level, demonstrating that a relatively rapid and facile reversible transition between the adherent and non-adherent state of cells can be regulated by SMC level. The reversibility with down-regulation indicates that SMC must be turning over at a significant rate. This turnover is presumably a significant feature of the participation of SMC in several biological processes, including metastasis, in which both adhesive and non-adhesive phenomena are important. A second example of the importance of SMC turnover occurs with blastocyst implantation, in which mucin must be rapidly removed from the uterine luminal epithelial cell surfaces at the appropriate time (33). Based on these observations, a model for the involvement for SMC during the metastatic process can be proposed. Up-regulation of SMC expression in cells in the solid tumor causes tumor cell detachment from adjacent cells and extracellular matrix in the primary site. This may lead to the penetration of these cells into the lymphatics, blood circulation, and body cavities. The non-adhesive nature of the SMC-expressing phenotype allows the cells to be transported to distant organs without adhering to neighboring tissues. Later, down-regulation of SMC results in the exposure of binding sites for endothelial cell selectins for adhesion of the tumor cell to the endothelial cell wall prior to extravasation. Alternatively, differential glycosylation on SMC may provide carbohydrate ligands for E-selectin to facilitate tumor cell adhesion to the activated endothelial cells without down-regulation of SMC. Thus, we propose SMC is a modulator of tumor metastasis, consistent with previous observations of a correlation of metastatic capability of 13762 sublines with expression of gp580 (ASGP-1) (24). This hypothetical metastasis model can be explored in vivo using cancer cell lines with inducible expression of SMC. Tumorigenicity and metastatic behavior of SMC-transfected cancer cell lines injected into mice are currently under investigation.

Acknowledgment—We thank Dr. Kathleen Kelly (National Institutes of Health) for providing the inducible expression vector puhHID10-3 and the transactivator plasmid ITA.

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