Sialomucin Complex, a Heterodimeric Glycoprotein Complex

EXPRESS AS A SOLUBLE, SECRETABLE FORM IN LACTATING MAMMARY GLAND AND COLON*

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Ascites 13762 rat mammary adenocarcinoma cells express abundantly on their cell surfaces a heterodimeric glycoprotein complex composed of a sialomucin ascites sialoglycoprotein (ASGP)-1 and a transmembrane subunit ASGP-2. The latter, which contains two epidermal growth factor-like domains, binds the receptor tyrosine kinase p185

The complex, which is encoded on a single gene (3), contains a highly glycosylated mucin subunit, ASGP-1, that is tightly, but noncovalently, linked to the membrane via the transmembrane glycoprotein ASGP-2 (4, 5). Biosynthesis studies showed that it is synthesized as a large (~300 kDa) polypeptide precursor that is cleaved to the two subunits early in its transit to the cell surface (6). The mucin ASGP-1 consists of a polypeptide of ~220 kDa bearing a heterogeneous collection of O-linked, sialic acid-rich oligosaccharides (7) and has been implicated in the metastasis (8) and resistance to killing by natural killer cells (9) of 13762 cells.

The complex is particularly interesting because of the structure of the transmembrane subunit ASGP-2, which appears to have functional activities other than linking the mucin to the membrane (4). ASGP-2 contains an 80-kDa polypeptide with ~17 N-linked oligosaccharides (10). Molecular cloning and sequencing identified seven domains of ASGP-2 (11): two N-glycosylated hydrophilic domains, two EGF-like sequences (EGF-1 and EGF-2), a non-EGF-like cysteine-rich domain, a transmembrane domain, and a short cytoplasmic domain. The presence of the EGF-like domains in these highly proliferative 13762 cells suggested the possibility of an autocrine growth factor-like activity associated with ASGP-2 (4). In support of this hypothesis, both EGF-1 and EGF-2 contain all of the consensus residues found in EGF-like sequences of proteins known to possess growth factor activity (11). Using an insect cell expression system, ASGP-2 has been shown to bind to the extracellular domain of the receptor tyrosine kinase p185

Sialomucin complex (SMC),‡ also called ASGP-1/ASGP-2, is expressed at very high levels (>10⁶ copies/cell) on the surface of metastatic 13762 ascites rat mammary adenocarcinoma cells (1, 2). The complex, which is encoded on a single gene (3), contains a highly glycosylated mucin subunit, ASGP-1, that is tightly, but noncovalently, linked to the membrane via the transmembrane glycoprotein ASGP-2 (4, 5). Biosynthesis studies showed that it is synthesized as a large (~300 kDa) polypeptide precursor that is cleaved to the two subunits early in its transit to the cell surface (6). The mucin ASGP-1 consists of a polypeptide of ~220 kDa bearing a heterogeneous collection of O-linked, sialic acid-rich oligosaccharides (7) and has been implicated in the metastasis (8) and resistance to killing by natural killer cells (9) of 13762 cells.

In an effort to understand the physiological functions and identify potential target tissues of ASGP-2, we have screened a

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1 The abbreviations used are: SMC, sialomucin complex containing ASGP-1 and ASGP-2; ASGP, ascites sialoglycoprotein; EGF, epidermal growth factor; mAb, monoclonal antibody; ELISA, enzyme-immunosorbent assay; PAGE, polyacrylamide gel electrophoresis; NGS, normal goat serum; PBS, Dulbecco’s phosphate-buffered saline without calcium; ABTS, 2,2’-azino-di-[3-ethylbenzthiazoline sulphonate]; PCR, polymerase chain reaction.

2 K. L. Carraway III, E. A. Rossi, M. E. Carvajal, P. M. Guy, D. Lorenzo, L. C. Cantley, R. A. Cerione, N. L. Fregien, C. A. C. Carraway, and K. L. Carraway, submitted for publication.
variety of rat tissues for the expression of ASGP-2 using mAbs elicited by immunization with SMC. ASGP-2 expression was found to be particularly abundant in lactating mammary gland and colon. In contrast to its cell surface expression in the ascites tumor cells, ASGP-2 is a secreted product in mammary gland and colon. Immunological and biochemical analyses demonstrated both membrane-associated and soluble forms in both mammary gland and milk but only a soluble form in the colon. The soluble form arises due to the absence of its COOH-termini.

Materials and Methods

Cells, Microvilli, and SMC Preparation—The MAT-B1 and MAT-C1 ascites sublines of the 13762 rat mammary adenocarcinoma were maintained by weekly passage as described previously (13). SMC used for immunizations and immunoblot analyses (10) and microvilli used as a positive control for immunoblot analyses (14) were prepared from MAT-C1 ascites cells as described previously.

Production of mAbs—Female Balb/c mice were immunized intraperitoneally twice with 50 µg of SMC emulsified with 250 µl of TitreMax Adjuvant (Vaxcel, Inc., Norcross, GA). On days 14 and 28 post-immunization, mice were injected with the same emulsion. The mouse with the best titre by ELISA was given a final injection in the tail vein with 50 µg of SMC in 100 µl of PBS on day 50. On the third day after the final injection, activated splenocytes were isolated and fused with p3x63Ag8 myeloma cells (ATCC, CRL 1580), using the procedures outlined by Harlow and Lane (15). For splenocyte isolation the spleen was aseptically dissected, and the splenocytes were teased out into 50 ml of PBS using a cell sieve dissociator (Sigma) and washed twice with PBS. The splenocytes were mixed with 4 × 10^7 p3x63Ag8 myeloma cells, and the cells were pelleted by centrifugation. Polyethylene glycol 1500 (1 ml of 50%) was slowly added to the combined cell pellet at 37°C. Following a 3-min incubation at 37°C, the polyethylene glycol cell suspension was slowly diluted with PBS at 37°C over 15 min to 50 ml. The cells were pelleted and resuspended in 200 ml of HAT medium (20% heat-inactivated fetal bovine serum, 5% p388D1 conditioned medium, 100 µM hypoxanthine, 400 nM aminopterin, 16 µM thymidine). The p388D1-conditioned medium was prepared from a confluent culture of p388D1 cells that had been treated for 2 days with 450 µM 6-thioguanine medium (Sigma) supplemented with 2% heat-inactivated fetal bovine serum and stimulated with 5 µg/ml Esherichia coli lipopolysaccharide for 2 days. The hybridomas were plated at 100 µl/well into twenty 96-well tissue culture plates that were fed with HAT (100 µl/well) after 7 days. On day 9 post-fusion, the hybridomas were screened by ELISA for production of anti-SMC antibodies. From each tissue culture well, 50 µl of conditioned medium was transferred to corresponding wells of ELISA plates that were coated with SMC at 1 µg/well. Anti-SMC antibodies were detected with a goat anti-mouse IgG-alkaline phosphatase conjugate. Optical densities (405 nm) of greater than 0.1 after 60 min of incubation were selected as positive.

Immunoblotting—As a secreted glycoprotein in COS-7 cells, the construct pDNA3/C12Swasmadebythedestructionof135basesatthe3′-endoftheASGP-2codingsequencefromthepcDNAI/C12MvectorandsubsequentlyclonedintothepcDNA3vector(Invitrogen). In addition to the pre-existing amino-terminal truncation, this deletion results in a 45-amino acid residue carboxyl-terminal deletion that removes the transmembrane and cytoplasmic domains and allows secretion of the glycoprotein. COS-7 cells were transiently transfected with pDNA3/C12Swasm celebrating the secreted form of ASGP-2. The resultant recombinant protein was harvested from tissue culture-conditioned medium.

Other Antibodies Used in This Study—Anti-ASGP-2 rabbit polyclonal antibody used for immunoprecipitations has been described previously (6). A mAb to an underdetermined F-cell antigen, which does not cross-react with rat and was used as a control for non-specific immunofluorescence, was kindly provided by Dr. Martin Flanjnik (University of Miami School of Medicine, Miami, FL). The anti-C-pep antibody is a rabbit polyclonal antibody that was raised against a synthetic peptide (NH2-CSMNKFSYPDSEL-COOH) from the COOH-terminal cytoplasmic domain of ASGP-2, amino acids 714–728. The peptide synthesis and antibody production were performed by Quality Controlled Biochemicals Inc.

Immunoblotting—SDS-PAGE was performed under reducing conditions using 6% polyacrylamide gels and the mini-Protein II system (Bio-Rad). Resolved proteins were transferred to nitrocellulose filters, which were subsequently blocked with 5% nonfat dry milk in Tris-buffered saline with 0.5% Tween 20. Following a 1-h incubation in primary antibody dilution in 1% bovine serum albumin/Tris-buffered saline with 0.5% Tween 20, 0.5% bovine serum albumin/Tris-buffered saline, the filters were incubated in peroxidase-conjugated goat anti-mouse IgG (Promega) diluted 1:100,000. The signal was detected with the Renaissance Enhanced Chemiluminescence kit (DuPont NEN).

Immunoprecipitations—Anti-ASGP-2 and anti-C-pep immunoprecipitations were performed with 20 µl of protein A-agarose (Sigma) and 20 µl of antisera. Anti-ASGP-1 immunoprecipitations were conducted with 20 µl of anti-IgM agarose (Sigma) and 20 µl of asacites fluid. All immunoprecipitations were rotated overnight at 4°C and washed a minimum of six times (10–20 min) in the appropriate buffer. Bound proteins were released by boiling in SDS-PAGE sample buffer.

Pulse-Chase Studies of Sialomucin Synthesis in 13762 MAT-B1 Cells for Analyzing Antibody Specificity—Ascites 13762 MAT-B1 cells (1 × 10^6) were washed three times with PBS and incubated in prelabeling medium (Cys- and Met-free RPMI 1640 supplemented with 10% dialyzed serum) for 30 min at 37°C. The cells were metabolically labeled with 2 mCi of [35S]Cys/Met in 9 ml of prelabeling medium for 10 min at 37°C. After washing with prelabeling medium, one half of the cells was immediately prepared for immunoprecipitation, whereas the other half was prelabeled at 37°C for 80 min in RPMI 1640 supplemented with 10% fetal bovine serum before preparation for immunoprecipitation. For each sample, cells were boiled for 1 min in 2 ml of 2% SDS. Lysates were homogenized with a 23-gauge needle and syringe and diluted with 10 ml of 2.5% Triton X-100, 6 µl EDTA, 100 µM NaCl, 60 µM Tris-HCl, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, 1 µM pepstatin (all protease inhibitors from Sigma). The lysates were clarified by centrifugation at 20,000 × g, and 1.5-mL aliquots were immunoprecipitated with nonimmune rabbit serum, anti-ASGP-2 polyclonal anti-serum, anti-ASGP-1 mAb 11C5, anti-ASGP-1 mAb 15H5, or nonimmune mouse IgM. The immunoprecipitates were washed six times (10–20 min) with Tris-buffered saline containing 0.1% Triton X-100, 0.02% SDS, 5 µl EDTA.
and then boiled in 40 μl of SDS-PAGE sample buffer. Immunoprecipitations were performed using SDS-PAGE and immunoblotting.

**Immunofluorescence of Lactating Mammary Gland Sections**—Mammary gland was dissected from post-partum Fischer 344 rats and fixed in 4% para-formaldehyde/PBS for 3 h at 4°C and then incubated overnight in 0.6 M sucrose. Samples were frozen in Tissue-Tek® O.C.T. embedding compound (Miles Inc., Elkhart, IN) with liquid N₂-cooled isopentane. 4-μm frozen cross-sections were prepared with a cryostat and mounted on microscope slides. Mounted slides were incubated in acetone for 10 min at 4°C and then washed twice for 10 min with PBS. All of the following steps were performed at room temperature with incubations in a humidified chamber using 100 μl of solution. The sections were incubated with 50 μl glycine (preadsorbed against rat serum, Sigma) diluted 1:100 in 1% bovine serum albumin/PBS. After a rinse and three 15-min washes, the sections were incubated with a 1:100 dilution of extravidin/TRITC (Sigma) for 30 min. Coverslips were applied with Gel/Mount signal saver after a rinse and three 15-min washes.

**Immunoperoxidase Staining of Colon Sections for Localization Studies**—Colon tissue was dissected from adult Fischer 344 female rats. Some rats were treated with either scopolamine or carbachol (Sigma) by intraperitoneal injection of 20 μg of the drug 5 min prior to sacrifice. Colon sections were prepared in 4% paraformaldehyde infused with 1/5 of the volume of 0.5 M LiCl overnight in 0.6 M sucrose. All samples were frozen in Tissue-Tek® O.C.T. embedding compound with liquid N₂-cooled isopentane. 4-μm frozen cross-sections were prepared with a cryostat and mounted on microscope slides. Mounted slides were treated with acetone for 10 min at 4°C and then washed twice for 10 min with PBS. All of the following steps for immunoperoxidase staining were performed at room temperature, with incubations in a humidified chamber using 100 μl of solution, unless otherwise stated. Endogenous peroxidase activity was reduced by incubating in 1% H₂O₂ in MeOH. The slides were rinsed and washed twice for 10 min in PBS, then incubated with 30 min in 10% NGS/PBS. Following two 5-min washes, the samples were incubated with primary antibody diluted in 3% NGS/PBS overnight at 4°C. The samples were rinsed and then washed three times for 10 min. The sections were incubated with a 1:300 dilution of a goat anti-mouse IgG-horse radish peroxidase conjugate (Promega) diluted in 3% NGS/PBS for 60 min. Following a rinse and three 10-min washes, the sections were incubated with Stable DAB (Research Genetics, Huntsville AL) substrate solution for 15 min. The sections were counterstained with 0.1% eosin in PBS for 1 min. The sections were dehydrated by ethanol and coverslips were applied with glycerin.

**Preparation and Biochemical Analysis of Tissue and Milk Samples**—Tissues dissected from female Fischer 344 rats were pulverized with a mortar and pestle in liquid N₂ and stored as a powder at −80°C. Fresh rat whole milk was diluted 1:4 with 25 mM HEPES, pH 7.4 (containing proteinase inhibitors: 1 mM phenylmethylsulfonyl fluoride, 1 mM kallikrein-inactivating unit/ml aprotinin, 1 mM leupeptin, 1 mM pepstatin) and homogenized with a 23-gauge needle and syringe. The diluted serum was isolated from the cream and insoluble material following centrifugation for 30 min at 15,000 × g and 4°C.

For immunoblotting, tissue powders were solubilized directly in SDS-PAGE sample buffer, and milk sera were diluted 1:2 in 2 × sample buffer. For quantification of total protein, powders were solubilized in 0.5% SDS, boiled, and then clarified by centrifugation at 12,000 × g. Protein concentrations were determined by A₂₈₀ using a protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA). Protein concentrations of MAT-B1 cell lysates and milk sera were determined similarly.

**Immunoprecipitations**—Powders were solubilized in 3 ml of lysis buffer (0.5 mM EDTA, 60 mM Tris-HCl, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 1 kallikrein-inactivating unit/ml aprotinin, 1 mM leupeptin, 1 mM pepstatin) using a probe sonicator. The homogenates were brought to 5% sucrose and fractionated by centrifugation at 600 × g for 10 min, 12,000 × g for 30 min, and 100,000 × g for 90 min. The pellets of each centrifugation and the 100,000 × g supernatant fluid were analyzed by anti-ASGP-2 immunoblotting. The pellets were resuspended from which they were pelleted and 2.5 μl of each sample was analyzed.

For velocity sedimentation analysis, the supernatant fluid of the 100,000 × g centrifugation (100S) was loaded onto a 5–20% sucrose gradient in PBS and centrifuged in a SW 40 rotor for 16 h at 23,000 RPM and 4°C. An equivalent (100S) sample was brought to 0.5% Triton X-100 and loaded onto a similar gradient that contained 0.1% Triton X-100. Each gradient was collected in 13 × 1-ml fractions from the bottom, and 5 μl of each sample was analyzed by anti-ASGP-2 immunoblotting.

**Enzyme Assays**—Alkaline phosphatase activity in milk and lactating mammary fractions was measured with p-nitrophenyl phosphate as a substrate. Fractions were diluted 1:5000 in substrate solution (1 mg/ml p-nitrophenyl phosphate, 0.5 mM MgCl₂, 1 mM diethanolamine, pH 9.8) and A₄₅₀ readings were taken after a 30-min incubation at room temperature. Lactoperoxidase activity in mammary fractions was measured using 2.2′-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) as a substrate. Fractions were diluted 1:5000 in ABTS solution (Boehringer Mannheim), and A₄₀₅ readings were taken after a 10-min incubation at room temperature.

**RT-PCR**—Total RNA was isolated from ascites 13762 MAT-C1 cells or lactating mammary gland using TRI REAGENT® (Molecular Research Center, Inc., Cincinnati, OH), and 1 μg was reverse transcribed with avian myeloblastosis virus reverse transcriptase (Promega) following the supplier’s suggested protocol. The polymerase chain reaction was performed in a PTC-100 programmable thermal controller (MJ Research Inc., Watertown, MA) with 25 cycles (1 min at 94°C, 30 s at 60°C, and 30 s at 72°C). PCR primer names and sequences are as follows: SL20 (5'-CCCTTCCTCAA- CAACTGCAGT-3'); KL20 (5'-CAGAACCATTCTCCGTCCGT-3'); SR20 (5'-CGCATGCTCAAGTCTCTCAG-3'); CR20 (5'-GGAAACTTGT- TCACTGAGACG-3'); and CR20 (5'-GATTTTCAGGTGACGCTGCCC-3'). PCR reactions were conducted with three primer sets: set X used primers SL20 and SR20; set Y used primers KL20 and CR20; and set Z used primers KL20 and CR20.

**RESULTS**

**Production of Hybridomas and Characterization of mAbs**—mAbs specific for ASGP-1 and ASGP-2 were produced to assess their expression and localization in normal tissues and tumors. Spleen cells from mice immunized with SMC (ASGP-1/ASGP-2) were fused to myeloma cells, and the resulting hybridomas were screened for anti-SMC antibodies by ELISA. Of the 1920 wells into which the fusion mixture was plated, approximately 1500 contained viable colonies after 1 week of selective growth in HAT medium, and 315 wells produced condition medium that yielded A₄₀₅ values >0.1 in the ELISA. Conditioned medium from the 48 wells yielding the highest signals (A₄₀₅ > 0.300) were screened by immunoblot analysis. Of these, 30 mAbs specifically recognized ASGP-2 and nine mAbs stained ASGP-1.

Ascites fluids generated from 10 of the anti-ASGP-2-secreting hybridomas were assayed by immunoblot analysis using purified SMC and three recombinant ASGP-2 deletion mutants to generate a gross epitope map (Fig. 1). The three deletion mutants were C4A683, which is missing the COOH-terminal 45 amino acids containing the transmembrane and cytoplasmic domains; N4A53, which is missing the NH₂-terminal 53 amino acids; and N3A53-C4A683, which is missing both the NH₂-terminal and COOH-terminal domains. Fig. 1A shows immunoblots with one mAb (4F12) of five that recognized only full-length ASGP-2 and C4A683. This staining pattern suggests that these five mAbs are specific for epitopes that are located within the 53 amino acids; and N3A53-C4A683, which is missing both the NH₂-terminal and COOH-terminal domains. Fig. 1A shows immunoblots with one mAb (4F12) of five that recognized only full-length ASGP-2 and C4A683. This staining pattern suggests that these five mAbs are specific for epitopes that are located within the 53 amino acid residues of the NH₂ terminus of ASGP-2. Fig. 1B shows immunoblots of one mAb (13C4) of five that recognized all of the three deletion mutants, suggesting specificity for an epitope located between amino acid residues 53 and 683. None of the mAbs tested showed specificity for the COOH-terminal 45 amino acids. The isotype of each of the 10 anti-ASGP-2 mAbs was determined to be IgG1 using an isotyping kit (Boehringer Mannheim).
Four anti-ASGP-1 mAbs (15H5, 11C5, 9D6, and 2E6) are IgMs, and anti-ASGP-1 mAb 15H10 is an IgG1. All of the hybridomas express kappa light chains. As shown for 11C5 in Fig. 2A, the anti-ASGP-1 mAbs specifically bind to a very high Mr band in immunoblots of 13762 MAT-B1 whole cell lysates. To verify that the anti-ASGP-1 antibodies are reacting with ASGP-1, we performed immunoprecipitation experiments on ascites lysates with both anti-ASGP-2 and anti-ASGP-1 antibodies. A band co-migrating with ASGP-1 was immunoprecipitated with anti-ASGP-2 (Fig. 2B), and ASGP-2 was immunoprecipitated using anti-ASGP-1 (Fig. 2C).

Because ASGP-1 is a mucin that contains large amounts of O-linked carbohydrates, the epitopes for the anti-ASGP-1 antibodies could contain carbohydrate. The nature of the epitope was investigated by pulse-chase analysis. Biosynthesis of ASGP-1 and ASGP-2 proceeds through high Mr precursor forms pSMC-1 and pSMC-2, which are not significantly O-glycosylated (6). These precursors are recognized by polyclonal anti-ASGP-2 (6) but should not be recognized by antibodies whose epitopes contain O-linked carbohydrate. In contrast, antibodies against O-linked carbohydrate epitopes should recognize mature, glycosylated ASGP-1, but not precursor. Anti-ASGP-2 monoclonal antibodies, of course, should recognize the precursors and mature ASGP-2 but not ASGP-1. For these experiments ascites 13762 MAT-B1 cells were metabolically pulse-chase labeled with [35S]Met + [35S]Cys in vitro and lysed with SDS after a 10-min pulse or a 10-min pulse and an 80-min chase. Cell lysates were immunoprecipitated with anti-ASGP-1 mAbs or anti-ASGP-2 polyclonal antibody. As predicted, polyclonal anti-ASGP-2 immunoprecipitated the SMC precursors, seen after the 10-min pulse, and mature ASGP-2 after the chase (Fig. 2D). Anti-ASGP-2 mAb 4F12 similarly immunoprecipitated the precursor forms from lysates of 10-min pulse-labeled cells and mature ASGP-2 from lysates of cells after 90 min (data not shown). In contrast, the anti-ASGP-1 mAbs did not immunoprecipitate the precursors. They immunoprecipitated only mature ASGP-1, observed after the 80-min chase (Fig. 2D). These data indicate that the anti-ASGP-1 mAbs recognize epitopes that are created by post-translational modifications, strongly suggesting that their epitopes contain O-linked oligosaccharides.

**Immunoblot Analysis of Tissue Homogenates**—Rat tissues were analyzed by SDS-PAGE and immunoblotting using four different anti-ASGP-2 mAbs (4F12, 1G5, 16A2, and 13C4), each of which showed the same pattern of reactivity. Results are shown for two different tissue surveys using mAb 4F12 in Fig. 3. Of the tissues screened, ASGP-2 expression was prominent in the intestine, trachea, uterus, and lactating mammary gland. The anti-ASGP-2-staining band in each of the strongly positive tissue homogenates displayed a similar SDS-PAGE migration to that of 13762 ascites cells, with estimated Mr values between 120 and 145. The broad signal for ASGP-2 is attributed to the heterogeneity of glycosylation, as observed for the 13762 ascites cell glycoprotein (10). The discrete, minor band observed for the urinary bladder (Fig. 3A) may be due to a low level of expression or to nonspecific staining. Recently,
ASGP-2 has been shown to be present in the lung (16) at levels that were too low for detection by direct immunoblot. ASGP-2 was detected when polyclonal anti-ASGP-2 immunoprecipitates from lung homogenates were immunoblotted with mAb 4F12 (data not shown).

ASGP-2 Expression in the Mammary Gland—ASGP-2 is the transmembrane subunit of SMC in the 13762 mammary adenocarcinoma. Because ASGP-2 is present in normal mammary tissue at higher levels than in many other tissues (Fig. 3A), ASGP-2 expression in 13762 adenocarcinoma cells, lactating mammary gland, and milk. SDS-solubilized samples of ascites 13762 MAT-B1 cells, cultured (13 passages) MAT-B1 cells, lactating mammary gland, and milk were loaded for SDS-PAGE as indicated at the top of the figure. Total protein loaded in each lane is indicated at the bottom. Anti-ASGP-2 immunoblotting was performed using mAb 4F12, and the signal was detected with ECL. The positions of molecular mass standards are indicated.

ASGP-2 Expression in the Mammary Gland—ASGP-2 is the transmembrane subunit of SMC in the 13762 mammary adenocarcinoma. Because ASGP-2 is present in normal mammary tissue at higher levels than in many other tissues (Fig. 3B), we compared the expression levels of ASGP-2 in the 13762 MAT-B1 subline from both ascites tumors and cultured (13 passages) cells with that in the lactating mammary gland. The $M_r$ of ASGP-2 in the lactating mammary gland is similar to that in the ascites tumor; however, the expression level is significantly lower (Fig. 3C). ASGP-2 is over-expressed by greater than 100-fold in the ascites tumor compared with the lactating mammary gland. As we have previously shown (17), ASGP-2 expression is significantly lower in MAT-B1 cells grown in culture compared with the in vivo ascites form. The level of ASGP-2 expression is about 5-fold greater in cultured MAT-B1 cells compared with the lactating mammary gland. Rat milk was similarly analyzed and shown to contain a substantial amount of ASGP-2 (Fig. 3C), estimated from immunoblot comparisons with isolated ASGP-2 to be $\approx 10 \mu g/ml$.

ASGP-2 Localization in Mammary Gland and Colon—The cell localization of ASGP-2 in lactating mammary gland was determined by immunofluorescence using anti-ASGP-2 mAbs. mAb 4F12 was used for indirect immunofluorescence because it very specifically stains ASGP-2 in immunoblots of lactating mammary gland homogenates (Fig. 3). Anti-ASGP-2 mAb 4F12 labels the apical portion of secretory epithelial cells of the gland (Fig. 4B). Some background fluorescence was seen within the epithelial cells (Fig. 4C), so it was not possible to determine if the ASGP-2 expression is restricted to the apical plasma membrane.

In the colon the staining was restricted to the apical side of secretory cells (Fig. 5). Two aspects of this staining suggested a localization in secretory granules. First, the entire volume of the cells between the apical membrane and nucleus appeared to be stained, as expected for secretory cells engorged with product to be secreted from granules. Second, cells of colon sections from rats treated with carbachol, an acetylcholine receptor agonist, and secretagogue for colonic cells (18) display a loss of anti-ASGP-2 staining with a concomitant morphological change, consistent with induced secretion (data not shown). Treatment with the acetylcholine receptor antagonist scopolamine as a control did not affect morphology or anti-ASGP-2 staining.

Expression of ASGP-2 in the Mammary Gland during Pregnancy—Homogenates of mammary gland from virgin, pregnant, and post-partum lactating rats were analyzed by anti-
ASGP-2 immunoblots to investigate the changes in ASGP-2 expression level during pregnancy (Fig. 6). One problem with this analysis was the presence of a co-migrating band recognized by second antibody (Fig. 6B). Quantitative subtraction of this interfering band indicated that ASGP-2 is essentially absent from virgin gland but increases dramatically around mid-pregnancy (Fig. 6, A and C). Its expression is maximal in the mammary gland of late pregnant (day 17) and post-partum rats.

Biochemical Characterization of ASGP-2 from Lactating Mammary Gland and Colon—In 13762 cells ASGP-2 is present as a complex with ASGP-1. Therefore, milk serum was analyzed by immunoperoxidase labeling with mAb 4F12 (A) or a control mAb specific for a frog T cell antigen (B). Sections were counterstained with hematoxylin and eosin. Photographs show the distal colon at 125× magnification.

Fig. 5. Immunoperoxidase localization of ASGP-2 in the distal colon. Consecutive sections (4 μm) of the distal colon were analyzed by immunoperoxidase labeling with mAb 4F12 (A) or a control mAb specific for a frog T cell antigen (B). Sections were counterstained with hematoxylin and eosin. Photographs show the distal colon at 125× magnification.

Fig. 6. Time course of expression of ASGP-2 in the mammary gland during pregnancy. SDS-solubilized samples of virgin (V), pregnant (3, 5, 11, and 17 days), and lactating (L) mammary tissue were loaded (10 μg/lane wet weight) for SDS-PAGE as indicated at the top of the figure. A, immunoblot with anti-ASGP-2 mAb 4F12. B, immunoblot without primary antibody, showing a nonspecific contaminating band. C, plot of relative ASGP-2 expression levels during pregnancy. The bands from A and B were quantified by densitometry. The densities of the co-migrating contaminating bands (B) were subtracted from those of the anti-ASGP-2 immunoblot, and the differences were plotted.

Fig. 7. Co-immunoprecipitation of ASGP-1 and ASGP-2 from milk. Rat milk serum was diluted 10-fold in 0.2% Triton X-100 and immunoprecipitated with nonimmune serum (NIS), anti-ASGP-2 polyclonal antibody, nonimmune IgM (NI-IgM), anti-ASGP-1 mAb 15H5, or anti-ASGP-1 mAb 11C5 as indicated at the top of the panel. The immunoprecipitates and a milk serum sample (lane 1) were subjected to SDS-PAGE, and immunoblots were performed using anti-ASGP-1 mAb 11C5 or anti-ASGP-2 mAb 4F12 as indicated at the bottom of the panel. The positions of molecular mass standards are indicated. IP Ab, immunoprecipitating antibody.

180-kDa species exists in a co-precipitating complex with ASGP-2. In contrast, both the high Mₗ and 160–180-kDa species were immunoprecipitated with both anti-ASGP-1 mAbs 15H5 and 11C5, as demonstrated by immunoblotting with mAb 11C5. The results with anti-ASGP-1 mAb 15H5 immunoblots were identical (data not shown). The co-immunoprecipitation of ASGP-2 and ASGP-1 from milk was confirmed by immunoblot analyses of anti-ASGP-1 immunoprecipitates with anti-ASGP-2 mAbs (Fig. 7A). Immunoblots with mAbs 1G5 and 13C4 showed the same results (data not shown) as mAb 4F12 shown in Fig. 7A. These data indicate that ASGP-2 in the lactating mammary gland and milk exists in a complex that is
Similar to SMC of the 13762 tumor cells.

Similar results were obtained for the colon. For this analysis we used the IgG anti-ASGP-1 mAb 15H10, which specifically binds a protein that co-migrates with ASGP-1 (data not shown). This very high Mr protein is also specifically stained with mAb 15H10 in anti-ASGP-2 immunoprecipitates but not in nonimmune immunoprecipitates (data not shown), confirming that ASGP-1 and ASGP-2 are present in a complex in the colon.

Evidence for a Soluble, Truncated Form of ASGP-2—Because ASGP-2 in the ascites tumor cells has been characterized as an integral transmembrane glycoprotein, we expected it to be associated with the milk fat globule membrane in milk. To test this hypothesis, we fractionated homogenized milk serum by ultracentrifugation and compared the distribution of ASGP-2 with that of alkaline phosphatase (AP). The bands from each centrifugation were quantified by densitometry (ASGP-2) and compared with the AP activity in each fraction. Bars representing the percentage of the total in the pellet and supernatant fluid (Sup.) from centrifugations lacking Triton X-100 as well as those containing Triton X-100 (Tx. Pellet and Tx. Sup.) are labeled.

![Fractionation of ASGP-2 in milk.](image)

**FIG. 8.** Fractionation of ASGP-2 in milk. A, milk serum was centrifuged at 100,000 × g in the presence (+ Triton) or the absence (− Triton) of 0.5% Triton X-100, and the pellets (P), supernatants (S), and unfractionated total (T) were analyzed by anti-ASGP-2 (mAb 4F12) immunoblotting. B, comparison of the ASGP-2 distribution with that of alkaline phosphatase (AP). The bands from A were quantified by densitometry (ASGP-2) and compared with the AP activity in each fraction. Bars representing the percentage of the total in the pellet and supernatant fluid (Sup.) from centrifugations lacking Triton X-100 as well as those containing Triton X-100 (Tx. Pellet and Tx. Sup.) are labeled.

To address the issue of the origin of the two forms of ASGP-2, lactating mammary tissue was analyzed by subcellular fractionation using differential centrifugation to determine the distribution of ASGP-2 in the gland. Lactating mammary tissue was homogenized by hypotonic lysis and sonication. The homogenates were fractionated by consecutive centrifugations at 600 × g, 12,000 × g for 30 min, and 100,000 × g for 90 min. The pellets of each centrifugation (12 P, 100 P) and the supernatant fluid of the 100,000 × g centrifugation were analyzed by

![Velocity sedimentation analysis of ASGP-2 in lactating mammary gland.](image)

**FIG. 9.** Subcellular fractionation of ASGP-2 in lactating mammary gland. Homogenates of lactating mammary tissue were subjected to sequential centrifugations of 600 × g for 10 min, 12,000 × g for 30 min, and 100,000 × g for 90 min. A, anti-ASGP-2 immunoblot analysis of the pellets of each centrifugation (0.6 P, 12 P, and 100 P) and the 100,000 × g supernatant fluid (100S) with mAb 4F12. B, the ASGP-2 bands from A were quantified by densitometry (hatched bars) and compared with the distributions of alkaline phosphatase (filled bars) and lactoperoxidase (shaded bars).

![Velocity sedimentation analysis of ASGP-2 in lactating mammary gland.](image)

**FIG. 10.** Velocity sedimentation analysis of ASGP-2 in lactating mammary gland. The supernatant fluid of a 100,000 × g centrifugation was fractionated in 5–20% sucrose gradients in the presence (+) or the absence (−) of 0.1% Triton X-100. Fractions were collected from the bottom (fraction 1) of the gradient and analyzed by immunoblotting with anti-ASGP-2 mAb 4F12.
anti-ASGP-2 immunoblotting (Fig. 9A). The majority of ASGP-2 was recovered from the 100,000 × g supernatant (100S). This was measured by densitometry to be 71% of the total ASGP-2 recovered. In a parallel fractionation of MAT-B1 13762 ascites cells, the distribution was 40% in 100S, 31% in 100 P and 29% in 12 P (data not shown). Because ASGP-2 from the ascites cells is membrane-bound, these results suggest that our sonication procedure for homogenization is creating small vesicles that are not sedimenting at 100,000 × g.

Two types of analyses were used to address the distribution of ASGP-2 further: comparisons with known markers and sucrose density gradient centrifugation. First, the distribution of ASGP-2 from the homogenized mammary gland was compared with those of alkaline phosphatase and lactoperoxidase (Fig. 9B). Alkaline phosphatase is a membrane protein in the lactating mammary gland, as described above, whereas lactoperoxidase is in the soluble fraction. The specific activity of alkaline phosphatase than alkaline phosphatase (Fig. 9B), preimmune rabbit serum (preimmune; lane 0), or a peptide antibody specific for the cytoplasmic domain of ASGP-2 (anti-C-pep; lane 1), as indicated. Lane 2, anti-C-pep immunoprecipitation from the resulting supernatant fluid from the immunoprecipitation in lane 1. Lanes 3a and 3b, anti-ASGP-2 and anti-C-pep immunoprecipitations, respectively, from the resulting supernatant fluid from the immunoprecipitation in lane 2. The immunoprecipitates were analyzed by immunoblotting with anti-ASGP-2 mAb 4F12.

ASGP-2 is expressed in the ascites cells as an integral membrane protein with a transmembrane domain near the COOH terminus. A soluble secreted form of ASGP-2 should not have the transmembrane and cytoplasmic domains. Therefore, there should be an isoform of ASGP-2 without the COOH-terminal transmembrane and cytoplasmic domains. To determine whether such an isoform exists, a rabbit polyclonal antiserum was raised against a synthetic peptide (NH₂-CSMNKFSYPLDSEL-COOH) from the COOH-terminal cytoplasmic domain of ASGP-2, amino acids 714–728. This antisera (anti-C-pep antibody) was used to immunoprecipitate ASGP-2 from mammary gland and colon homogenates and 13762 MAT-B1 cell lysates. The immunoprecipitates were analyzed by SDS-PAGE and immunoblotting with anti-ASGP-2 mAb 4F12. As shown in Fig. 11, full-length ASGP-2 can be efficiently immunoprecipitated with the anti-C-pep antibody from both the mammary gland and ascites 13762 MAT-B1 cell lysates. The supernatant fluids from these immunoprecipitations were then depleted of full-length ASGP-2 by two additional rounds of immunoprecipitation with the anti-C-pep antibody. No full-length ASGP-2 could be detected in the third immunoprecipitates of either sample (Fig. 11), indicating that they had been depleted of ASGP-2, which has the COOH-terminal intracellular domain. Immunoprecipitation of the second round supernatants with polyclonal anti-ASGP-2 (whole molecule) showed that the mammary gland sample still contained ASGP-2, which could not be immunoprecipitated with the anti-C-pep antibody, suggesting that the carboxyl-terminal portion of the protein was absent from these molecules. There was no detectable ASGP-2 in the MAT-B1 samples following two rounds of immunoprecipitation with the anti-C-pep antibody. These data indicate that as predicted the soluble isoform of ASGP-2 found in the mammary gland does not contain a transmembrane domain, whereas all of the ASGP-2 expressed in the ascites cells has a transmembrane domain and is not secreted. By this analysis
the truncated form of the ASGP-2 is about 25% of the total, which agrees well with the amount observed in the fractionation studies. In contrast, no ASGP-2 was immunoprecipitated from colon homogenates with the anti-C-pep antibody (data not shown), indicating that all of the colon ASGP-2 is truncated, consistent with the absence of a membrane form by gradient analysis and the localization in secretion granules.

Analysis of ASGP-2 Transcripts by RT-PCR—The two simplest mechanisms that would generate the soluble isoform of ASGP-2 are: 1) alternative splicing of ASGP-2 mRNA resulting in a protein that lacks a transmembrane sequence; 2) specific proteolytic cleavage of a transmembrane precursor. To investigate the potential for the former, cDNA was made from RNA of ascites 13762 cells, lactating mammary gland, and adult colon and subjected to PCR analysis (Fig. 12). Three overlapping sets of oligonucleotide primers were designed to examine the 3' end of the ASGP-2 mRNA. If an alternative splicing mechanism is responsible for the generation of the soluble isoform, one would expect to observe amplimers, which differ in size from those of the 13762 cells. However, the amplimers generated from lactating mammary gland and colon cDNA co-migrated with that from the 13762 cells for each of the three primer sets (Fig. 12). For each of the primer sets, a single amplimer was detected from each tissue. These data argue against alternative splicing as the mechanism that produces the soluble ASGP-2 isoform.

DISCUSSION

ASGP-2, the transmembrane subunit of the cell surface sialomucin complex in the 13762 rat mammary adenocarcinoma, is expressed in the secretory epithelial cells of lactating mammary glands and colon. It is secreted into milk in the mammary gland and released by a secretagogue in the colon. Its behavior in these tissues is thus quite different from that in the 13762 cells in which it is found as a membrane-associated, cell surface protein. However, the mammary gland, colon and ascites tumor glycoproteins do have some important common features. The Mr displayed by mammary and colon ASGP-2 in SDS-PAGE is comparable with that of the tumor. The two appear to be antigenically indistinguishable; each of the mAbs generated against tumor ASGP-2 recognizes the glycoprotein from the two normal tissues. As found for the tumor (1), ASGP-2 is associated in a complex with ASGP-1 in the mammary gland, milk, and colon.

Three important differences have been found between ASGP-2 expression in these two normal tissues and the 13762 ascites tumor cells: 1) ASGP-2 is found at more than a 100-fold greater level in the 13762 ascites cells. Because ASGP-2 has been shown to be a ligand for the receptor tyrosine kinase p185 neu, this over-expression may contribute to the proliferative and metastatic properties of this tumor. 2) ASGP-2 is secreted from mammary epithelial cells into milk and from the colon cells upon secretagogue stimulation. Although a substantial fraction (~70%) of the secreted protein in milk is membrane-associated, fractionation comparisons with a milk fat globule membrane marker indicate that it is not primarily associated with the milk fat globule membrane. This conclusion is supported by fractionation studies of the mammary gland. These results suggest that the membrane-associated sialomucin complex is secreted by a different mechanism from the milk fat globule membrane. Milk is known to contain a second membrane fraction, the so-called skim milk membrane fraction (20). Our fractionation studies suggest that the sialomucin complex is associated with these membranes. Moreover, because the origin of these milk membranes is uncertain (21), sialomucin complex may provide a suitable marker for the investigation of the pathways contributing to their secretion. 3) A truncated, soluble isoform of ASGP-2 is present in the mammary gland and colon. This isoform, which is also secreted into milk, lacks the cytoplasmic domain.

Two possibilities for a mechanism for producing soluble ASGP-2 are by alternative splicing of mRNA or by a post-translational proteolytic modification. We examined the 3' half of the ASGP-2 mRNA by RT-PCR analysis. Because anti-ASGP-2 mAbs recognize colon ASGP-2 and their epitopes were mapped to regions amino-terminal (5') to the EGF-1 domain, we suspect that a such a splicing event would occur 3' of the EGF-1 sequence. Using primers that span this region, PCR amplifiers from a splice variant encoding a soluble isoform are expected to be of different size than those of the membrane bound isoform of the 13762 cells. Further, one would predict that such a soluble splice variant would be the dominant one in the colon, because only the soluble isoform is detected. However, with three primer sets that each spanned the 3' portion of the ASGP-2, a single amplimer was detected from mRNA of 13762 cells, lactating mammary gland and colon. For each primer set, the amplimers from all three cDNAs comigrated. Thus, we were unable to detect any splice variant that might be responsible for generating the soluble isoform, although a splice variant that removes a small exon and creates a reading frameshift and downstream stop codon might not be detected.

ASGP-2 expression is dramatically up-regulated in the mammary gland during pregnancy. We were unable to detect ASGP-2 in mammary gland homogenates from virgin or early pregnant rats. ASGP-2 increases in mid-pregnant (day 11 of 21) animals, where its expression is nearly 50% of that in later pregnant and lactating animals. Gene expression in the mammary gland is regulated by both hormones and the extracellular matrix, exemplified by studies on the milk proteins β-casein and whey acidic protein (22). Expression of β-casein, a "mid-phase" milk protein, is induced around day 10 of pregnancy in mice, whereas expression of whey acidic protein, a "late" milk protein, increases most between days 15 and 17 of pregnancy (23). Based on data presented here, we would classify sialomucin complex as a mid-phase milk protein, suggesting that its expression may be regulated similarly to the β-casein gene. The regulation of sialomucin complex expression in the mammary gland is currently being investigated. Its up-regulation during pregnancy and abundance in milk suggests a possible biological role as a milk component.

As a heterodimeric, bifunctional glycoprotein, the sialomucin complex could be playing a number of roles in milk. Milk mucins have been suggested to be involved in the protection of the newborn through the binding of microorganisms (21, 24). Two different types of milk mucins have been described (21). The best characterized is MUC1, the polymorphic epithelial mucin (4, 5, 25–27). The second milk mucin, variously called component A and MUCX, has a higher apparent Mr, is more heavily glycosylated, and has not been well characterized (21, 28). Based on the studies described here and comparative amino acid compositions (28, 29), ASGP-1 is a likely candidate for component A/MUCX. Component A was originally described as being associated with the human milk fat globule membrane (28). However, no milk mucins are found in the fat globule membranes of the rat (30). Thus, variations in the distribution of milk membrane mucins may be species-dependent.

The fact that ASGP-2 can bind the receptor tyrosine kinase p185 neu suggests a possible function as a growth regulator. Interestingly, the molar concentration of ASGP-2 in milk is about an order of magnitude higher than that of EGF (31). One of the primary targets of milk growth factors is the intestine of the neonate. Interestingly, ASGP-2 expression in the secretory...
cells of the colon arises at the time of weaning. Thus, ASGP-2 is available to influence intestinal physiology throughout the life span of the rat, either from the milk or endogenous sources. These observations suggest that the sialomucin complex may play a significant role in the intestine. Additional studies will be necessary to define that role.

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