Identification of a Novel Membrane Protein, HP59, with Therapeutic Potential as a Target of Tumor Angiogenesis

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ABSTRACT

CM101, a polysaccharide isolated from the culture medium of Group B streptococcus, a neonatal pathogen, targets pathological angiogenesis and inhibits tumor growth in mice and humans. CM101 also targets neonatal lung and adult sheep lung endothelial cells. A gene encoding a transmembrane protein that interacts with CM101 was isolated from a sheep lung endothelial cell cDNA library. The gene, termed sp55, encodes a 495-amino acid polypeptide. COS-7 cells transfected with a vector containing sp55 express the SP55 protein-bound CM101 in a concentration-dependent manner. Stably transfected CHO cells also bound CM101. The corresponding human gene, hp59, was isolated from a human fetal lung cDNA library and had a predicted identity to SP55 of 86% over 495 amino acids. HP59 protein was shown by immunohistochemistry to be present in the pathological tumor vasculature of the lung, breast, colon, and ovary, but not in the normal vasculature, suggesting that the protein may be critical to pathological angiogenesis. The hp59 gene and/or the HP59 protein was not expressed in a variety of normal tissues, but was significantly expressed in human fetal lung, consistent with the pathophysiology of Group B streptococcus infections in neonates. Mice immunized with HP59 and SP55 peptides showed significant attenuation of tumor growth. Immunization effectively inhibited both the tumor angiogenesis and vasculogenesis processes, as evidenced by lack of both HP59- and CD34-positive vessels. These results and the immunohistochemistry data suggest a therapeutic potential for the CM101 target protein HP59 both as a drug target and as a vaccine against pathoangiogenesis.

INTRODUCTION

GBS is a neonatal pathogen. Infection of human newborns with GBS is associated with severe respiratory distress, pulmonary hypertension, pulmonary edema, and circulatory collapse, which are referred to as GBS pneumonia or early-onset disease (1). The symptoms of early-onset disease persist even after the bacterial infection is eliminated by antibiotics, suggesting that there is an involvement of an extracellular toxin (2). The putative agent responsible for these symptoms, GBS toxin, has been partially purified from the culture medium of GBS (3) and from the urine of infected neonates (4). In its purified form, GBS toxin is referred to as CM101 (5).

CM101 is a polysaccharide with an approximate molecular weight of 300,000 (5). The pathophysiology associated with neonatal pneumonia could be readily duplicated in a sheep model when CM101 was infused in picomolar quantities (3). Neither serotype or group-specific polysaccharides nor cell-wall extracts or yeast-derived media polysaccharides had any effect in the sheep model (3). Damage to sheep lung vasculature by GBS toxin infusion was evidenced by changes in hemodynamics (6) very similar to those seen in the human neonates. These observations led to the hypothesis that CM101 bound to embryonic target proteins present in neonatal lung neovascularature and induced an inflammatory response, leading to respiratory failure (7). It was also proposed that similar target proteins were present in tumor neovascularure, which becomes a target for CM101 (8). Indeed, CM101 administered i.v. at μg/kg dosages was demonstrated to inhibit tumor growth in both tumor-bearing mice (8, 9) and humans (10, 11). The Phase I data suggested a rapid complement (C3)-activated cytokine-driven inflammatory response, which led to tumor shrinkage in some patients (10). It

Received 3/9/01; revised 8/1/01; accepted 8/1/01. 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.

1 This work was supported by grants from CarboMed, Inc. and AngioPath Inc., in which B. D. W., H-P. Y., C. C., D. L. P., R. S. L., and C. G. H. have a financial interest, and by the Vanderbilt Ingram Cancer Center.
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The abbreviations used are: GBS, Group B streptococcus; IHC, immunohistochemistry; SLEC, sheep lung endothelial cell; WB, wash buffer; FACS, fluorescence-activated cell sorting; 5’RACE, 5’ rapid amplification of cDNA end; mAb, monoclonal antibody; ScFv, single-chain fragment variable; UTR, untranslated region; KLH, keyhole limpet hemocyanin; CFA and ICFA, complete and incomplete Freund’s adjuvant, respectively; T-PBS, PBS containing 0.1% Triton X-100; vWF, von Willebrand factor; PKC, protein kinase C; CKII, casein kinase II.
was demonstrated in a mouse model that CM101 bound to tumor neovasculature and activated complement by the alternative pathway (12). Targeted endothelial cells with C3b bound to CM101 are immediately lysed by leukocytes, which all express C3b receptors. The C3b receptor also has a broad spectrum lectin-binding domain (13). CM101, by binding multivalently to the target proteins on the endothelial cell, converts the latter to a bacterial mimetic with lethal consequences to the cell. This initial event induced leukocyte infiltration of the tumor within 5 min (12). The overall response induced by CM101 led to neovascularitis followed by tumor apoptosis (14), which was also evident in cancer patients (15, 16).

It was demonstrated early that GBS toxin/CM101 did not affect wound healing in the sponge model (17). A study on the effect of a wound on tumor angiogenesis and the effect of CM101 on the two pathological conditions showed that CM101 ablated the tumors and facilitated wound healing with full tensile strength within 7 days (18). It was also shown that CM101 inhibited collagen-induced rheumatoid arthritis by inhibiting pathological hypoxia-driven angiogenesis without affecting physiological angiogenesis in the growth plate (19). Collectively, these data led to the conclusion that CM101 is antipathoangiogenic (12) and warranted the expressional cloning of the target membrane glycoprotein that CM101 binds to in neonatal and, later in life, in pathological neovasculature.

We report here the expressional cloning of a protein that is a target for CM101. The CM101 target protein gene was identified from a sheep lung cDNA library and encodes a protein, SP55, with 7 (20) or 12 (21) putative transmembrane domains, depending on the program used for prediction. The corresponding gene cloned from a human fetal lung cDNA library, hp59, also encodes a 7- or 12-transmembrane domain protein. IHC analysis using antibodies to HP59 or SP55 detected expression of HP59 only on the endothelial cells of human neonatal lung and on vasculature in pathological tissues from mice and humans, consistent with the observed biological activity of CM101. The therapeutic potential was indicated by the application of HP59 and SP55 peptides as vaccines in a mouse tumor model. The immunized mice showed a significant attenuation of tumor growth and inhibition of angiogenesis and vasculogenesis.

MATERIALS AND METHODS

Primary Culture of Sheep Lung Endothelial Cells. Pieces of primary lung tissue from a 7-week-old sheep were obtained from Professor H. W. Sundell at the Department of Pediatrics of Vanderbilt University (Nashville, TN). The tissue was minced in HBSS containing 10 mM HEPES buffer (pH 7.4; Life Technologies, Inc., Gaithersburg, MD), 1% penicillin/streptomycin, and 0.1% gentamicin, and were cultured in complete DMEM with low glucose supplemented with 5% bovine calf serum, 5% Nu serum, 1% penicillin/streptomycin, 0.1% gentamicin, 5 units/ml heparin, and 50 μg/ml endothelial cell growth factor supplement (Life Technologies) at 37°C. Clones of sheep lung endothelial cells were identified by their cobblestone morphology (22) and transferred into 24-well tissue culture plates, using cloning rings. Confluent cultures were detached by 1X pancreaticin (Life Technologies) and transferred to 60-mm tissue culture plates or T-25 tissue culture flasks. Cultures were then propagated in 100-mm plates. A total of 10^6 cells were used for isolation of mRNA.

Complement (C3) Binding Assay and Flow Cytometric Analysis. SLECs were grown in sheep lung medium as described above. Cells were washed once with HBSS and detached by incubation with 1X pancreatin in PBS for 30 min at 37°C. After collection in 1.5-ml microcentrifuge tubes (1×10^6/tube), the cells were pelleted in a microcentrifuge at 1000×g for 5 min. The cells were then washed once with HBSS (Life Technologies) plus 5% fetal bovine serum (referred to as WB). This was followed by incubation with 100 μl of CM101 solution (10 μg/ml diluted in PBS containing Ca^{2+} and Mg^{2+}) at room temperature for 60 min with slow shaking. Cells were then pelleted, washed once with the WB, resuspended in 100 μl of PBS containing normal human serum or heat-inactivated human serum (1:40 dilution), and incubated at room temperature for 60 min. After centrifugation, cells were again washed with WB and resuspended in 100 μl of FITC-conjugated sheep antihuman C3 antibody [20 μg/ml diluted in PBS (Binding Site, San Diego, CA)]. After a 1-h incubation at room temperature, the cells were pelleted, washed twice with WB, and resuspended in 1% paraformaldehyde for FACS analysis. FACS was done at the Flow Cytometric Laboratory of the Veteran’s Administration Hospital (Nashville, TN).

Isolation of mRNA and Construction of a SLEC cDNA Library. Poly(A)^+ RNA was isolated from 9.2×10^7 sheep lung endothelial cells (passages 8 and 9), using a mRNA isolation kit (FastTrack 2.0 Kit) provided by Invitrogen (Carlsbad, CA). A total of 16 μg of poly(A)^+ RNA was obtained. A total of 2.5 μg of mRNA was submitted to Invitrogen to construct a SLEC cDNA library. Poly(A)^+ RNA was oligo(dT)-primed (with NotI restriction site) and converted into double-stranded cDNA. After a BstXI/EcoRl adapter was added, the cDNA was unidirectionally cloned into the BstXI and NotI sites of pCDNA3.1(+) (Invitrogen). Escherichia coli Top10F (Invitrogen) was used as a host strain for amplification. A total of 5.38×10^6 primary clones were generated. The library was amplified by plating cells onto 50 large LB agar plates containing ampicillin (100 μg/ml). The plates were scraped and aliquoted so that each aliquot represented 10 plates. DNA was purified by Qiagen Max columns (Qiagen, Valencia, CA).

Biotinylation of CM101. The polysaccharide CM101 (25 μg/ml) was biotinylated at the reducing end by incubation with 5 mM biotin-hydrazide (Pierce, Rockford, IL) and 100 mM cyanoborohydride (Sigma Chemical Co., St. Louis, MO) in 0.1 N sodium acetate buffer (pH 4.0) for 16 h at room temperature, followed by dialysis of the final product against water. The product was analyzed by high-pressure liquid chromatography and quantitated by Dioderay analysis to contain 1 mol biotin/mol of CM101.

Screening of CM101 Candidate Receptor Gene from cDNA Library. The expression of the CM101 target protein receptor gene from the cDNA library was assayed in an ELISA. Five μg of plasmid DNA from each pool of the SLEC cDNA library were used to transfect COS-7 cells. The transfected cells were cultured in 96-well tissue culture plates (Falcon) for transient expression. Each well contained ~20,000 transfected cells in DMEM (Life Technologies). COS-7 cells transfected with
pCDNA3.1(+) were used as controls. The medium was carefully removed after 3 days of growth at 37°C, and the cells were rinsed three times with WB. The cells were then incubated with biotinylated CM101 (50 μg/well; 1–1.5 μg/ml) in PBS containing Ca^{2+} and Mg^{2+} at room temperature for 1 h. Unbound biotinylated CM101 was removed, and the cells were rinsed three times with WB. The cells were incubated with streptavidin-β-galactosidase solution (1:1000 dilution in WB; 50 μg/well) at room temperature for 1 h. After removal of the streptavidin-β-galactosidase solution, each well was rinsed three times with WB. The cells were then incubated with p-Nitrophenyl-β-galactopyranoside (50 μg/well; 1 mg/ml in substrate buffer; Ref. 12) was added and incubated at 37°C. The absorbance at 405 nm was measured at 1 and 20 h. The cells from wells that gave the highest absorbance were harvested. Plasmid DNA was isolated as described (23). Plasmid DNA was amplified in E. coli, and another transfection was performed. This enrichment procedure was repeated eight times. The number of transfected cells loaded in each well was gradually decreased, and untransfected cells were added to give a total of 20,000 cells per well. The last ELISA assay was performed with each well containing only 1–10 transfected cells. Cells from wells with the highest absorbance were harvested. DNA was isolated and amplified in E. coli. COS-7 cells were transfected with DNA from single clones and were individually assayed for expression by ELISA. The COS-7 cells transfected with the clone pFU102 showed the highest binding of CM101; DNA from this clone was sequenced and yielded a 2.1-kb insert.

On the basis of sequence analysis, pFU102 contains a 2.1-kb insert encoding a partial integral membrane protein. Using a part of pFU102 as a probe, we isolated a different clone, pSL55, from a commercial sheep fetal lung library (Stratagene, La Jolla, CA) according to the company’s protocol. The insert in pSL55 was ~2.8 kb (including the entire sequence of pFU102); however, further analyses revealed that it was not a full-length clone. Attempts to identify a full length of the clone from both libraries failed. Through 5’RACE, a PCR product was digested with SalI and PstI and cloned into the SalI and PstI double-digested pBluescript II KS(+) (Stratagene), yielding pCR111. 5’RACE was performed with a 5’RACE kit (Life Technologies), which yielded the full-length clone.

**Northern Blot Analyses.** Poly(A)^+ mRNA (~1.0 μg each) was isolated from sheep lung, liver, and kidney by a standard protocol (Pharmacia, Uppsala, Sweden). Poly(A)^+ RNA was fractionated on a formaldehyde gel and subsequently transferred to nylon membrane as described (23). Prehybridization (2 h) and hybridization (overnight) were carried out in 50% formamide at 42°C. The blot was washed in 1× SSC-0.1% SDS, 0.2× SSC-0.1% SDS and 0.2× SSC-0.1% SDS for 20 min at 25°C, 25°C, and 42°C, respectively. The nylon membrane was exposed to a Kodak film at ~80°C.

Northern blots of human tissues were purchased from Clontech (Palo Alto, CA) and Invitrogen. Northern blotting was done by the procedure described above.

**Construction of Expression Clones pCD55H and pCD55.** Plasmid pSL55 was digested with Avai and XhoI, and an ~2.7-kb Avai-XhoI fragment was isolated. Two oligonucleotides (5’-CATGAGTCCCCGGTTGAGACTT-AGCC-3’ and 5’-TCGGGGCTAAATCGCAAAACCGGGGACTTCCATAGGTAC-3’), which covered the missing NH2 terminus of sp55 and contained a KpnI site, were synthesized and annealed. The annealed oligonucleotides and Avai-XhoI fragment of pSL55 were cloned into the KpnI-XhoI site of pCDNA3.1/HisA (Invitrogen) by a triple-ligation procedure to yield pCD55H. By the same method, two annealed oligonucleotides (5’-CATGAGTCCCCGGTTGAGACTT-AGCC-3’ and 5’-TCGGGGCTAAATCGCAAAACCGGGGACTTCCATAGGTAC-3’) and an Avai-XhoI fragment were cloned into the KpnI and XhoI-digested site of pCDNA3.1(+) to yield pCD55. Constructs were verified by DNA sequence analysis.

**Protein Purification and Immunoblot of SP55.** COS-7 cells were transiently transfected with pCDNA3.1 (control vector) and pCD55H encoding the sp55 gene. The transfected cells were incubated at 37°C for 3 days and scrambled off the plates with lysis buffer (Promega, Madison, WI). Whole-cell extracts from confluent cultures were used for purification of the 6-His-tagged recombinant protein by Ni-charged affinity chromatography under denaturing conditions (Invitrogen). Columns were precipitated with 10% trichloroacetic acid, washed with ethanol, dissolved in SDS loading buffer, and resolved by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes and subsequently immunostained with a 6-His tag antibody (Invitrogen) or with a rabbit antibody (Pab56) developed against a synthetic peptide based on the CM101 receptor sequence.

**Stable Transfection of CHO-K1 Cells with the sp55 Gene.** The sheep gene sp55 was cloned into expression vector pCDNA3.1(+), termed pCD55, with the G418-resistant gene (Invitrogen). pCD55 and the expression vector alone were linearized with PvuI for the stable transfection. The CHO-K1 cell line for transfection was purchased from American Type Culture Collection and maintained according to original protocol. Initially, a dose-response curve with the selective antibiotic marker G418 was established for the selection of stably transfected cell clones. CHO-K1 cells were cultured to ~80% confluence in 100-mm Petri dishes and collected in 0.02% EDTA. Cells were resuspended into calcium-magnesium-free HBSS (Life Technologies) and pelleted after their total cell number was determined. The cells were resuspended at 1.3 × 10^7 cells/ml of ice-cold sterile potassium PBS buffer (137 mM KCl, 2.7 mM NaCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 0.5 mM MgCl₂). Cell aliquots of 300 μl (3.9 × 10⁶ cells) were transfected with 5 μg of pCD55 or pCDNA3.1+ linearized DNA by electroporation in 0.4-cm electrode cuvettes at 200 V and 975 μF capacitance on a Gene Pulser II (Bio-Rad, Hercules, CA). Cells were transferred to 10 ml of medium containing 15% serum in gelatin-coated 100-mm Petri dishes and cultured for 48 h. The medium was replaced with a medium containing the selective antibiotic G418 at the lethal concentration of 1 mg/ml established initially for the nontransfected cells. Resistant clones were transferred separately into 24-well tissue culture trays with 1 ml of growth medium and G418 and were cultured for further expansion.

**Single-Chain Antibodies to CM101-7A3-ScFv.** The 7A3 mAb (12) was developed into a ScFv recombinant antibody. Briefly, mAb 7A3 mRNA was purified from 6 × 10⁶ hybridoma cells with a mRNA purification kit (QuickPrep; Amersham-Pharmacia). First-strand cDNA was obtained with a First Strand cDNA synthesis kit (Amersham-Pharmacia) and
random hexamers. First-strand cDNA was amplified by PCR using AmpliTaq DNA polymerase and synthetic oligonucleotide primers specific for the 5’ and 3’ ends of mouse antibody variable heavy or light chain genes. Variable heavy and light chain PCR products were gel band-purified and assembled into a ScFv product via overlap PCR with a 15-amino-acid (Gly4 Ser) linker. ScFv were PCR-amplified with primers encoding SfiI and NotI restriction sites; the PCR products restriction-digested with SfiI and NotI were then ligated to the pCANTAB5E phagemid expression vector (Amersham-Pharmacia). Ligated product was used to transform E. coli TG1 cells. Transformed cells were plated onto 2× YT agar medium containing 2% glucose and 100 µg/ml ampicillin and grown overnight at 30°C. A modified colony-lift (24) and BA85 nitrocellulose filters (Schleicher & Schuell) coated with CM101 (~1–2 µg/filter) were used to identify colonies producing ScFv reactive with CM101. For large-scale recombinant antibody production, colonies producing ScFv reactive with CM101 were used to inoculate 1- to 4-liter flasks containing 2× YT agar medium containing 2% glucose and 100 µg/ml ampicillin. Cells were grown overnight at 30°C with shaking and centrifuged. The pellet was resuspended in 2× YT containing 1 mm isopropyl-1-thio-β-D-galactopyranoside and ampicillin, and the cells were grown overnight at 30°C with shaking. Cells were pelleted via centrifugation and resuspended in 40 ml of 0.2M Tris-HCl (pH 8.0), 0.5 mM EDTA, 0.5 M sucrose (Tris-EDTA-sucrose) and 60 ml of 0.5 M sucrose (Tris-EDTA-sucrose) for every liter of bacterial culture. Resuspended cell pellets were placed on ice for 1 h and centrifuged. The supernatant containing the soluble ScFv from the bacterial periplasmic extract was removed, transferred to polypropylene centrifuge tubes, and stored at −70°C until needed. ScFv were purified from periplasmic extract with the Amersham-Pharmacia Recombinant Phage Antibody System purification module.

FACS Analyses of Stably Transfected CHO-K1 Cells. Cultures of pCD55 and vector-alone stably transfected clones were detached in trypsin-EDTA, washed in WB, resuspended in F12 medium, and cultured in suspension for 2 h at 37°C. Cells were washed twice in HBSS and incubated with 5 µg/ml CM101 in HBSS for 30 min at 37°C. Cells were washed twice in HBSS and blocked with 50 µg/ml mouse IgG in HBSS; 5 µg/ml anti-CM101 single-chain antibody conjugated with FITC was added and incubated for 30 min at 37°C. The cells were pelleted, washed twice with WB, and resuspended in 1% paraformaldehyde for FACS analysis. FACS was performed at the Flow Cytometric Laboratory of the Veteran’s Administration Hospital (Nashville, TN).

Cloning of CM101 Candidate Receptor Gene from a Human Library. SP55 showed 30–39% identity with two hypothetical proteins from Candida elegans (C38C10.2 and ZK512.6; Ref. 25). On the basis of the C. elegans consensus sequences from sp55 and the two C. elegans proteins, two degenerate oligonucleotides (5’-CGGATCCGCGCCGNNATGCAYRSHTSSTG-3’ and 5’-GGAATCNGGGCRATKTCNARKRTT-3’) were synthesized and used to PCR-amplify a human fetal lung cDNA (Invitrogen). PCR products were digested with EcoRI and BamHI and cloned into the EcoRI and BamHI double-digested pBluescript II KS(+) fragment. A positive clone, PCR52, was identified through an open probe screening. PCR52 had a 720-bp insert containing a high homology sequence to the sp55 gene. This DNA was used as a probe to screen a human fetal lung cDNA library (Clontech), and two positive phage clones containing large inserts were isolated and subcloned into the EcoRI site of pBluescript II KS(+) (pLH21 and pLH461). On the basis of their sequences, both pH21 and pH461 contained a sequence identical to that of PCR52, but were not full-length clones. pH461 had the complete 3′UTR, including the poly(A) tail. Through 5′RACE, a PCR product was digested with SaI and EcoRI and cloned into the SaI and EcoRI double-digested site of pBluescript II KS(+), yielding pH132, which contained the partial gene up to the NH2 terminus.

Isolation of Genomic DNA Corresponding to the NH2 Terminus and Promoter Region of the Human CM101 Candidate Receptor Gene. A human genomic library (BAC library) was commercially screened (Research Genetics, Huntsville, AL) with the 720-bp EcoRI-BamHI fragment of PCR52 as a probe. Ten positive clones were identified, digested by PstI, and probed by oligonucleotide P55-AS1 (antisense; 5′-GACGCTTACGTGAGCAGGGT-3′), which corresponded to the 5′ region of pH132 (hp59 region). All genomic clones contained a positive 3.0-kb PstI fragment. This 3.0-kb PstI fragment was subcloned into the PstI site of pKSII(+) and yielded the genomic clone H317. On the basis of genomic sequencing analysis of H317, sense oligonucleotides PC-S0 (5′-GGACAAATATGCCGCAATGCAGC-3′), PC-S2 (5′-GTCTGGTGAAGCCCTCCC-3′), PC-S3 (5′-CAGGAGGCGGAGACGGTGCTGC-3′), and PC-S4 (5′-CAGGAGGCGGAGACGGTGCTGC-3′) were synthesized and used, each with P55-AS1 (antisense), as primers for PCR using human fetal lung cDNA as a template. Except for the pair PC-S0 and P55-AS1, all primer pairs were able to produce PCR products. PCR products were cloned into pGEM-T Easy vector (Life Technologies) and screened. A cDNA clone, S2H1, which contained a larger insert (385 bp) covering the 5′ end of the hp59 gene, was identified from the PCR product amplified from the PC-S2 and P55-AS1 primers.

Preparation of mAbs to HP59-derived Peptides. BALB/c mice received initial injections of 100 µg of a synthetic peptide H1 (amino acids 50–63; Fig. 3) conjugated with KLH (Sigma Genosys, Inc., Woodlands, TX) in CFA followed after 2 weeks with 100 µg of HP59 peptide in ICFA (Sigma). Four weeks after the initial injection, the mice were boosted with 100 µg of the peptide H1 alone. The boost was repeated twice at 1-week intervals. Antibody titers were determined by ELISA. Three days prior to cell fusion, 50 µg of H1 peptide was injected i.v. into the mice as a final boost. Spleen cells from mice immunized with HP59 peptides were fused with X63-Ag8-653 murine myeloma cells as described by Fu and Carter (26). Fused cells are plated into 96-well culture plates (Costar Corporation, Cambridge, MA) and subsequently screened for IgG-recognizing synthetic HP59 peptides. Positive cultures were then expanded, subcloned, and screened for hybridomas producing mAbs for IHC on frozen or fixed tissue sections. The mAbs (Hab1) were affinity-purified by HP59 peptide affinity chromatography (26).
Preparation of Polyclonal Antibodies to SP55-derived Peptides. The SP55-derived peptide P56 (amino acids 9–35; Fig. 3) and the same peptide conjugated with KLH by Sigma Genosys were used to immunize rabbits. New Zealand White rabbits were immunized first with 300 μg of the peptide conjugated with KLH and mixed with CFA. Two weeks after the initial injection, the rabbits were boosted with 300 μg of the same peptide, without KLH, mixed with ICFA. The boost was repeated three times at 2-week intervals. The antibody titer was determined by ELISA. Blood was collected at the completion of immunization, and the serum was separated and stored at −80°C. The anti-P56 antibody (Pab56) was purified by affinity chromatography with the synthetic peptides. Binding of the purified antibody to the P56 peptide was confirmed by ELISA.

IHC. High-grade papillary carcinoma of the ovary, poorly differentiated adenocarcinoma of the colon, invasive mammary carcinoma of the breast, and poorly differentiated non-small cell carcinoma of the lung were subjected to IHC analysis. Paired human tumor and normal tissues were each embedded in paraffin, and 6-μm sections were prepared. Sections were deparaffinized by immersing slides in xylene at room temperature twice for 5 min. The slides were then transferred to fresh absolute alcohol and rehydrated through a graded alcohol series, ending with PBS. Sections were then treated with 0.1 M sodium borate buffer for 20 min and washed with PBS. Sections were then blocked with a biotin blocking system (Dako), washed with PBS, and blocked for 40 min with PBS containing 5% BSA and 5% normal goat serum. Ten μg/ml affinity-purified monoclonal antireceptor HP59 antibody in PBS was added to each section and incubated in a humidified chamber for 1 h at room temperature. The slides were washed three times with T-PBS and incubated with biotinylated goat antirabbit IgG (Dako) under the same conditions as described for the primary antibody. The slides were then washed three times with T-PBS. Streptavidin-FITC conjugate (Life Technologies) was added to the sections and incubated for 40 min in a humidified chamber. The slides were washed again with T-PBS, and rabbit antihuman vWF IgG (Dako; diluted 1:200) was added to the sections and incubated for 1 h. The slides were washed with T-PBS and incubated for 1 h with biotinylated goat antirabbit IgG (Jackson ImmunoResearch). After washing with T-PBS, a streptavidin-Cy3 conjugate (Amersham) was then added to the sections and incubated for 40 min at room temperature. The slides were washed with T-PBS, dehydrated, and mounted with coverslips.

Lewis lung tumors from control and immunized mice were fixed, paraffinized, and subjected to the above-described procedure except that primary antibodies to Pab56, Hb1, and vWF were used. The CD34 protocol required treatment with Retrieve All (Signet, Dedham, MA). The secondary antibodies were biotinylated, and streptavidin-horseradish peroxidase-3,3′-diaminobenzidine was used for development of the stain according to standard protocols (12).

CM101 Target Protein as Vaccine. Seven male and female mice were each immunized with 100 μg each of the SP55 peptides P56 (amino acids 9–35; Fig. 3) and P57 (amino acids 71–84) and the HP59 peptides H1 (amino acids 50–63), H2 (amino acids 112–125), and H3 (amino acids 8–28; Fig. 3) conjugated to KLH and emulsified with CFA. The immunization was repeated three times every 2 weeks with the KLH conjugates in ICFA. Five female and male control mice were immunized with CFA and ICFA. Mice were bled 1 week after the last immunization, and an absorbance of >2 was recorded for serum diluted 1:800 for Pab56 antibodies. Hb1 did not elicit a strong response. Lewis lung cell suspensions (10⁶ cells) in 3% agar were implanted s.c. in seven immunized male and five immunized female mice and in four male and four female mice immunized with CFA alone as controls. The mice were observed daily until the control tumors began to ulcerate, at which time mice were sacrificed and tissues, including tumors, were collected for histology and toxicology.

RESULTS

Complement-binding Assay and FACS Analysis of Sheep Lung Endothelial Cells. CM101 targets exclusively the endothelium of the sheep lung and induces an inflammatory response (3) through complement activation (12). The presence of CM101 target proteins in SLECs was established by FACS analysis of SLECs (1 × 10⁶) sequentially incubated with CM101, human serum containing C3, and FITC-labeled sheep antihuman C3 (SAHC3) antibody. FACS analyses showed that SLECs bound the fluorescently labeled sheep antihuman C3 antibody (FITC-SAHC3) in the presence of CM101 and C3 (Fig. 1a, shaded histogram), compared with the control without CM101 (Fig. 1a, open histogram). SLECs did not significantly shift in the presence of CM101 and heat-inactivated serum (data not shown).

Expression Cloning of the CM101-binding Protein. A cDNA library was constructed from the mRNA isolated from the SLECs that had previously been shown to bind CM101. COS-7 cells were transiently transfected with pools of cDNA in 96-well ELISA plates, and the cells were assayed for biotinylated CM101 binding. Wells with absorbance higher than that of the control cells transfected with the plasmid (pCDNA3.1) alone (Fig. 1b) were subjected to eight successive enrichments (see “Materials and Methods”) and selection by ELISA.

On the basis of the binding data, a unique clone was identified that contained a 2.1-kb insert (pFU102; Fig. 1c) which was predicted to encode part of a transmembrane protein. pFU102 had three methionines, the first at amino acid 57 in the open reading frame. However, DNA sequence analysis suggested that pFU102 was not a full-length clone. Screening of an additional sheep lung cDNA library, combined with a 5′ RACE, yielded pSL55 and PCR111 (Fig. 1c). On the basis of the sequences for pSL55 and PCR111, the mRNA for the CM101 candidate receptor gene from SLEC consisted of 2844 bp and was named sp55 (Fig. 1c).

The open reading frame of sp55 predicted a 495-amino acid protein with a calculated molecular mass of 55 kDa. The protein SP55 has seven potential transmembrane domains. On the basis of the seven-transmembrane domain profile (20), consensus sequences S/T-X-K/R for PKC phosphorylation sites (27) were identified at residue positions 73, 97, 254, 269, and 276. Consensus sequences S/T-X-X-E for CKII phosphorylation sites (27) were identified at residue positions 73, 79, 259, and 452. Potential N-glycosylation sites (N-X-S/T; Ref. 28) were predicted at positions 276, 278, and 280.
identified at positions 59, 71, 77, 95, 225, 302, and 357. In addition, a number of consensus sequences for myristoylation were also identified within the SP55 protein (data not shown). SP55 showed 30–39% identity to two hypothetical proteins in *C. elegans* and 25–39% identity with the sodium-inorganic phosphate cotransporters in humans, rabbits, and rats.

**Northern Blot Analyses.** The tissue distribution of the mRNA for the *sp55* gene was established by Northern blot
transmembrane domains. The gene hp59 showed a very significant shift for pCD55 transfected cells (Fig. 1g, shaded peak) compared with the vector-alone-transfected clone pCDNA3.1 (Fig. 1g, filled peak), demonstrating that the hp59 stably transfected cells bind CM101.

Cloning of a Human CM101 Candidate Receptor Gene from a Human Library. The sp55 gene from sheep showed some homology with two hypothetical proteins from C. elegans (25). On the basis of the alignment of SP55 with the two hypothetical proteins (data not shown), two degenerate oligonucleotides were synthesized and used for PCR of human fetal lung cDNA. A positive clone, PCR52, which contained a 720-bp insert, was identified among the PCR product clones (Fig. 2). The 720-bp EcoRI-BamHI fragment from PCR52 was used as a probe to screen a human fetal lung cDNA library. Two positive clones, pH21 and pLH461 (Fig. 2) were obtained. Through 5′ RACE, PHR132, which contained a partial gene, was obtained. Because of the unsuccessful isolation of the 5′ end by 5′ RACE, a human genomic library (BAC library) was screened with the 720-bp EcoRI-BamHI fragment of PCR52. After a positive clone was subcloned, plasmid H317, which contained ~3.0 kb of the human genomic DNA, was isolated. On the basis of the genomic sequence of H317,5 a few primers were made and used for PCR with human fetal lung cDNA as a template (see “Materials and Methods”). A cDNA clone, S2H1, which contained ~385 bp, was isolated (Fig. 2).

On the basis of the complete sequences of pLH21, pLH461, PHR132, and H317, it was concluded that the human CM101 candidate receptor gene consisted of 2930 bp, and was termed hp59. The gene hp59 was predicted to encode a 536-amino acid protein with seven potential transmembrane domains (Fig. 3, underlined sequences). Like SP55, HP59 also has seven

5 C. Fu, N. D. Cetateanu, S. Bardhan, and C. G. Hellerqvist, unpublished observations.
potential N-glycosylation sites (N-X-T at positions 100, 112, 118, 136, 266, 343, and 398; Fig. 3, indicated with asterisks) and a number of possible myristoylation sites (data not shown). On the basis of a seven-transmembrane domain profile (20), such proteins can be classified into two subgroups. The first subgroup comprises proteins with an additional 41 amino acids at the NH2 terminus compared with SP55 and is detected only in vascular endothelium, whereas the NH2 terminus of the second subgroup is cleaved by dipeptidyl peptidase IV (DPPIV) and is not expressed in vascular endothelium. The consensus sequences for PKC phosphorylation were identified at residue positions 298 and at positions 120, 138, 310, and 317, respectively. The consensus sequences for CKII phosphorylation (S/T-X-X-E) were identified at residue positions 298 and at positions 120, 138, 310, and 317, respectively. The consensus sequences for cAMP-dependent protein kinase were identified at residue positions 298 and at positions 120, 138, 310, and 317, respectively. The consensus sequences for PKC phosphorylation were identified at residue positions 298 and at positions 120, 138, 310, and 317, respectively. The consensus sequences for cAMP-dependent protein kinase were identified at residue positions 298 and at positions 120, 138, 310, and 317, respectively. The consensus sequences for PKC phosphorylation were identified at residue positions 298 and at positions 120, 138, 310, and 317, respectively. The consensus sequences for cAMP-dependent protein kinase were identified at residue positions 298 and at positions 120, 138, 310, and 317, respectively.

Northern Blot Analysis. The distribution of CM101 candidate receptor gene in human tissues was determined by Northern blot analyses (Fig. 3). Lane 1: fetal liver; Lane 2: fetal muscle; Lane 3: fetal skin; Lane 4: fetal brain; Lane 5: fetal small intestine; Lane 6: bladder; Lane 7: spleen; Lane 8: kidney; Lane 9: placenta; Lane 10: pancreas; Lane 11: adult lung. These differences may be attributable to different half-lives of the mRNA and/or pathological conditions of the donor tissues.

IHC Staining. A large number of normal and tumor tissues were stained with either Hab1 or Pab56 (Table 1). Tumor vasculature in all tumors was positive for HP59. To validate that HP59 was present in the endothelium, the mouse mAb Hab1, which is specific for HP59, and polyclonal rabbit antihuman vWF, which is specific for endothelial cells, were used for IHC on paired human normal and tumor tissues. The Hab1 binding was amplified with FITC (green) and vWF binding with Cy3 (red). Collectively, these data demonstrate that HP59, the CM101 target protein, was not detectable in normal human ovary, colon, breast, or lung tissue, where vasculature stained only red for vWF, but was abundant in the endothelial cells of tumors from human ovary, colon, breast, and lung (Fig. 5), where endothelium stained yellow, demonstrating that HP59 and vWF colocalize in the pathological vasculature of the tumors and not in vasculature of the normal tissues. Vasculature from mouse tumors and rheumatoid arthritic tissue also stained (data not shown).

Vaccine Targeting Tumor Vasculature. When challenged with 104 Lewis lung tumor cells s.c., mice immunized with homologous peptides derived from the amino acid sequences predicted by the method of Klein et al. (20) to be transmembrane domains are underlined. * potential N-glycosylation sites. The sequences of hp59 and sp53 have been deposited at GenBank under AF244577 and AF24578, respectively. The consensus sequences for PKC phosphorylation were identified at residue positions 298 and at positions 120, 138, 310, and 317, respectively. The consensus sequences for cAMP-dependent protein kinase were identified at residue positions 298 and at positions 120, 138, 310, and 317, respectively. The consensus sequences for PKC phosphorylation were identified at residue positions 298 and at positions 120, 138, 310, and 317, respectively. The consensus sequences for cAMP-dependent protein kinase were identified at residue positions 298 and at positions 120, 138, 310, and 317, respectively. The consensus sequences for PKC phosphorylation were identified at residue positions 298 and at positions 120, 138, 310, and 317, respectively. The consensus sequences for cAMP-dependent protein kinase were identified at residue positions 298 and at positions 120, 138, 310, and 317, respectively. The consensus sequences for PKC phosphorylation were identified at residue positions 298 and at positions 120, 138, 310, and 317, respectively. The consensus sequences for cAMP-dependent protein kinase were identified at residue positions 298 and at positions 120, 138, 310, and 317, respectively.

Northern blot analyses. Human tissue blots were purchased from Invitrogen (A, B, and D) and Clontech (C). A, Lanes: 1, fetal brain; 2, fetal liver; 3, fetal lung; 4, fetal muscle; 5, adult lung; B, Lanes: 1, fetal heart; 2, fetal kidney; 3, fetal skin; 4, fetal small intestine; 5, adult lung; C, adult tissues. Lanes: 1, heart; 2, brain; 3, placenta; 4, lung; 5, liver; 6, muscle; 7, kidney; 8, pancreas. D, adult tissues. Lanes: 1, muscle; 2, uterus; 3, bladder; 4, kidney; 5, spleen; 6, lung. Two μg of mRNA were loaded in each lane. The blots were probed with a 253-bp BsrDI cDNA fragment (coding region of hp59) from PCR52, and β-actin (B-actin) was used as a control probe.

Fig. 4 Amino acid alignment of SP55 and HP59. Sequences predicted by the method of Klein et al. (20) to be transmembrane domains are underlined. * potential N-glycosylation sites. The sequences of hp59 and sp53 have been deposited at GenBank under AF244577 and AF24578, respectively.
HP59, a CM101 Target Protein

Table 1  Human tumor tissues positive for HP59 protein as detected with polyclonal Pab56 and monoclonal Hab1

<table>
<thead>
<tr>
<th>Human ovarian cancer</th>
<th>Human colon cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>High-grade papillary carcinoma</td>
<td>Poorly differentiated adenocarcinoma</td>
</tr>
<tr>
<td>Advanced ovarian papillary serous carcinoma</td>
<td>Poorly differentiated adenoma</td>
</tr>
<tr>
<td>Granulosa cell tumor</td>
<td>Invasive differentiated adenocarcinoma</td>
</tr>
<tr>
<td>Serous papillary tumor with borderline feature</td>
<td>Moderately differentiated adenocarcinoma</td>
</tr>
<tr>
<td>Papillary serous cystadenocarcinoma</td>
<td></td>
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<tr>
<td>Metastatic adenocarcinoma</td>
<td></td>
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<tr>
<td>Clear cell carcinoma</td>
<td></td>
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<tr>
<td>Poorly differentiated serous carcinoma</td>
<td></td>
</tr>
<tr>
<td>Poorly differentiated adenocarcinoma</td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>Human female breast cancer</th>
<th>Human lung cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infiltrating mammary carcinoma (NST&lt;sup&gt;a&lt;/sup&gt;)</td>
<td>Poorly differentiated NOS small cell carcinoma</td>
</tr>
<tr>
<td>NOS</td>
<td>Invasive poorly differentiated squamous cell carcinoma</td>
</tr>
<tr>
<td>Invasive mammary carcinoma (NST)</td>
<td>Infiltrating moderately differentiated squamous cell carcinoma</td>
</tr>
<tr>
<td>Infiltrating mammary carcinoma (high combined grade)</td>
<td>Poorly differentiated adenosquamous carcinoma</td>
</tr>
<tr>
<td>Invasive mammary (high combined grade)</td>
<td>Squamous cell carcinoma</td>
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</table>

<table>
<thead>
<tr>
<th>Human male breast cancer</th>
<th>Liver tumor</th>
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</thead>
<tbody>
<tr>
<td>Mammary carcinoma</td>
<td>Metastatic moderately differentiated adenocarcinoma</td>
</tr>
</tbody>
</table>

<sup>a</sup>NST, no special type; NOS, not otherwise specified.

toxicity or abnormality in the immunized mice, except that the liver and kidney tissues showed sparse infiltration of lymphocytes, which is typical for CFA immunization.

**IHC Analysis of the Lewis Lung Tumors.** The tumors dissected from HP59-immunized and control-immunized mice were subjected to IHC analysis with Hab1 and Pab56 to detect the HP59 mouse homologue CM101 target protein. CD34 staining was used to detect early vasculogenesis and vWF to also mark mature vessels. The tumors from control immunized mice (Fig. 7, A, C, E, and G) showed the following: for tumors, typical large vessels (29) positive for vWF (Fig. 7A); CD34-positive vessels (Fig. 7C) as evidence of vasculogenesis; and HP59- (Fig. 7E) and SP55-positive (Fig. 7G) endothelium as evidence for pathoangiogenesis. In contrast, the tumors from the mice immunized with HP59- and SP55-derived KLH-conjugated peptides showed the small vWF-positive vessels (Fig. 7B) typical of normal tissue (Ref. 29; Fig. 5) and a striking lack of CD34-positive vessels (Fig. 7D). However, CD34-positive single cells were present, and when these data were combined with the lack of HP59- (Fig. 7F) and SP55-positive (Fig. 7H) vessels, this supports the notion that the pathoangiogenesis and concomitant vasculogenesis processes were effectively inhibited in the immunized mice. The presence of only small vWF-positive vessels suggests that in the immunized mice, the s.c. Lewis lung tumor used existing normal vessels, possibly through intussusceptive vessel proliferation, for its nutritional needs (29).

**DISCUSSION**

In both preclinical and clinical studies, CM101 has been shown to inhibit tumor growth. The mechanism of inhibition of tumor growth has been shown to be the rapid specific binding (within 5 min) of CM101 to the tumor vasculature (12), followed by a C3-activated cytokine-driven inflammatory response (10, 16). CM101 is a bacterial polysaccharide, which as such is composed of repeating oligosaccharide units. The rapid binding in vivo detected by IHC after i.v. infusion of 200 fmol of CM101 (12) suggests a very high affinity of CM101 for the tumor endothelium. On the basis of the molecular weight and quantitative sugar composition, of which each CM101 molecule has ~150 repeat units, each unit could bind to the target protein with low affinity. However, cooperative binding would explain the high affinity in vivo.

We previously established that the pathophysiology of the respiratory distress syndrome seen in human neonates infected at birth with GBS could be mimicked in the sheep model with i.v. infusions of 25 pmol/kg CM101 (3). Thus, SLECs were cultured, shown to bind CM101 by FITC-labeled antihuman C3 antibody in the presence of CM101 and human C3, and used as a source for a cDNA library. Through a unique ELISA assay, the gene (sp55) encoding a target protein, CM101, was cloned from the SLECs.

COS-7 cells transfected with the sp55 gene showed significant binding to CM101, indicating that the protein SP55 has binding sites for CM101. Because the polysaccharide CM101 consists of a large number of repeat units, it has multiple binding sites for cross-linking the SP55 protein. Although we could demonstrate concentration-dependent binding of CM101 to the transfected cells (Fig. 1e), kDa values for CM101 binding to the target protein could not be determined because of the complex nature of this binding. The data from the FACS analysis of sp55 stably transfected CHO-K1 cells, using CM101 and an FITC-conjugated CM101-specific single-chain antibody, demonstrate a very significant binding of CM101 to the sp55-transfected cells compared with vector-alone-transfected cells (Fig. 1G).

The binding of CM101 to SP55 and HP59 is not a common carbohydrate protein interaction because there is no homology with lectins or enzymes associated with the biosynthesis or degradation of carbohydrates. The hydrophobic nature of
CM101 (5) suggests that CM101 interacts with a unique domain(s) on the target proteins HP59 and SP55, which we have not yet identified.

The uniqueness of the interaction is further corroborated by the fact that GBS is the only bacteria producing a component (CM101) that can induce an inflammatory reaction affecting neovascularure only in neonates but not in normal children and adults (1). We recently demonstrated that CM101 could be isolated from the urine in GBS-infected neonates but not from neonates with other diseases (4). Dextran, a 270-kDa polysaccharide, was used as a control polysaccharide with no effects in most of our in vivo studies with CM101 (8, 9, 12, 14, 17).

SP55 protein possesses 7 (20) or 12 (21) potential transmembrane domains, depending on the program used for prediction, and several N-glycosylation sites. On the basis of database searches at the time we first sequenced it, SP55 did not show high homology to a known protein, although it showed 30–39% identity to sodium-phosphate cotransporters in humans, rabbits, and rats, and to two hypothetical proteins from C. elegans. The pathophysiology of GBS is characterized by pulmonary hypertension (7). Interestingly, when SP55 was compared with G-protein-coupled receptors, the fourth transmembrane domain of SP55 showed significant identity (66%) to the second transmembrane domain of angiotensin II receptor (30), which is

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**Fig. 5** Immunolocalization of HP59 in sections of human tumor and normal human control tissues. Tissue sections were analyzed with Hab1. A, human ovarian tumor tissue section; B, normal human ovarian tissue section; C, human lung tumor tissue section; D, normal human lung tissue section; E, human colon tumor tissue section; F, normal human colon tissue section; G, human breast tumor tissue section; H, normal human breast tissue section.
HP59, a CM101 Target Protein

The gene hp59, which encodes the human CM101 target protein, encodes a 536-amino acid polypeptide that has an additional 41 amino acids in the NH$_2$ terminus compared with SP55, and like SP55, HP59 has several N-glycosylation sites as well as phosphorylation sites, including cAMP-dependent protein kinase, PKC, and CKII.

IHC data demonstrated that HP59 was present on the vasculature of all tumor tissues tested (Table 1), including lung, colon, ovary, and breast cancers of different stages, but not in the corresponding normal tissues. The double stains for HP59 and vWF (Fig. 5) confirm that the CM101 target protein is present in the tumor endothelium.

Hp59 message is present in different fetal tissues during development, but consistent with the pathophysiology of GBS disease in newborns, HP59 is present only in lung endothelium at birth and until day 5 after birth in term babies.$^6$

Although hp59 mRNA was detected on commercial blots of human adult pancreas, placenta, and kidney, HP59 protein was not detectable by IHC in those normal human tissues. These commercially available Northern blots were loaded with pooled mRNA from 10 individuals. If one individual had a diseased tissue, then it is possible that this tissue contaminated the particular pooled lot of mRNA. This could then yield a positive signal in that sample. This may be a plausible explanation for the positive lung signal seen on the Invitrogen blot but not on the Clontech blot. Furthermore, the clinical trials involving cancer treatment with CM101 showed no toxicity to normal vasculature (10, 11) and no signs of organ toxicity, supporting the IHC observations (31). This is also consistent with the lack of pathology associated with GBS in normal adults.

IHC analysis of placental tissue was also negative for HP 59. This is in agreement with the experience in delivery rooms, where women colonized with GBS in the placenta have no complications (32). Both mother and baby can have a CM101 concentration in the blood 100 times higher than (4) the dosage used in the clinical trials (10, 11, 31), and the mother will have no symptoms, but the baby initially will have severe respiratory distress (4).

The positive mRNA blots but negative IHC may represent a particular form of spliced mRNA not representing HP59 (33) or may be attributable to posttranscriptional mechanisms regulating gene expression in eukaryotes (34), all of which give a poor correlation between protein and mRNA abundance (35).

These results support that HP59 protein is a unique marker for neonatal lung vasculature and adult pathological angiogenesis. It should be noted that a 495-amino acid stretch of the HP59 protein is 100% identical to a recently described protein, called sialin (36). The hp59 genomic DNA, the reverse transcription-PCR product, and subsequent analysis, however, showed that HP59 has an additional 41 amino acids at the NH$_2$ terminus compared with Sialin. Sialin is suggested to be involved in sialic acid storage disease (36).

The isolated sialin gene (SLC17A5) is $\sim$2512 bp long, which is significantly shorter than hp59 (2930 bp long). On the basis of the alignment of hp59 and sialin (data not shown), sialin, unlike hp59, does not have a complete 3' UTR with a poly(A) tail. The sialin gene also has a different 5' end compared with hp59. Our Northern blot probe with either the coding region or the 3' UTR of the hp59 gene showed that the hp59 gene is not expressed in all tissues and that when expressed, it is only as one transcript in all tissues tested (Fig. 5). In contrast, the Northern blot analysis with the sialin gene surprisingly showed three transcripts (4.5, 3.5, and 1.8 kb; Ref. 36). The sialin gene was isolated with a 400-bp expressed sequence tag (EST) sequence from a human fetal library, whereas we isolated hp59 with a 700-bp probe based on homologous sequences from C. elegans and sp55. The EST sequence spans the 3' end of HP59 over the translated and untranslated domains. Sialin is predicted to be a lysosomal membrane protein with a 12 or 14 transmembrane configuration. However, no lysosomal targeting signal was found (36).

HP59 showed some identity to two hypothetical proteins from C. elegans. Because all proteins from C. elegans studied to date have been shown to play significant roles in development, one could expect that HP59 also has an important function in development in humans. The expression of HP59 in only pathological and neonatal vasculature suggests that HP59 is not a widely distributed protein and indicates that HP59 could play an essential role in embryonic angiogenesis and, later in life, in pathological angiogenesis.

The vaccine experiment used an immunogenic amino acid sequence that is present extracellularly if HP59 is present in a seven-transmembrane configuration. Mice immunized with SP55- and HP59-derived peptides developed high titers to the SP55-derived peptide, and tumor growth was significantly attenuated. Immunization with the HP59- and SP55-derived peptides led to inhibition of vasculogenesis, as indicated by the absence of CD34-positive vessels, and inhibition of pathological angiogenesis, as evidenced by the absence of HP59-positive vessels.

$^6$ C. Fu, H. Sundell, and C. G. Hellerqvist, unpublished observations.
The presence of small vWF vessels typical of normal tissue may suggest that the s.c. implanted tumor used existing normal vessels, possibly through intussusceptive growth (32).

IHC studies using the polyclonal Pab56 and the monoclonal Hab1 antibodies (11 of 15 amino acids identical) show that both antibodies detect a mouse homologous protein in pathological vasculature in tumors (Fig. 7, E and G), an observation made in numerous samples (data not shown). The low immune response to H1 suggests that H1 is more “self” to the mouse than is P56.

The homology between the mouse and human CM101 target proteins indicated by IHC of pathological vasculature from both species, the successful vaccination of the mice with a closely related peptide (>80% identity), and the obvious inhibition of new vessel formation supports that a human vaccine against pathoangiogenesis is possible. In ad-
dition, HP59 has a 41-amino acid sequence at the NH2 terminal that has no homology with any known protein, and the appearance of HP59 in pathological vasculature in adults suggests that HP59 can be developed as a drug and diagnostic target.

ACKNOWLEDGMENTS

We thank Drs. James V. Staros and Jim Price for advice and suggestions through the duration of this project and Dr. Carlos Artega for critical and valuable comments regarding the manuscript. We respectfully acknowledge the early contributions of Drs. Timothy Genster, Carlos Castillo, and Gerald York, and Judith Briggs to this project. We thank Pamela Chunn for the completion of this manuscript. Experiments were performed in part through use of the Vanderbilt University Medical Center Cell Imaging Resource (supported by Grants CA68485 and DK20593).

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Identification of a Novel Membrane Protein, HP59, with Therapeutic Potential as a Target of Tumor Angiogenesis

Changlin Fu, Smriti Bardhan, Nicolae D. Cetateanu, et al.

*Clin Cancer Res* 2001;7:4182-4194.

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