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*J Immunol* 2011; 186:2344-2354; Prepublished online 7 January 2011; doi: 10.4049/jimmunol.1000840 http://www.jimmunol.org/content/186/4/2344

Supplementary Material

http://www.jimmunol.org/content/suppl/2011/01/07/jimmunol.1000840.DC1.html

References

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Molecular and Functional Characterization of Mouse S5D-SRCRB: A New Group B Member of the Scavenger Receptor Cysteine-Rich Superfamily

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The scavenger receptor cysteine-rich superfamily (SRCR-SF) members are transmembrane and/or secreted receptors exhibiting one or several repeats of a cysteine-rich protein module of ~100 aa, named scavenger receptor cysteine-rich (SRCR). Two types of SRCR domains (A or B) have been reported, which differ in the number of coding exons and intradomain cysteines. Although no unifying function has been reported for SRCR-SF members, recognition of pathogen-associated molecular patterns (PAMPs) was recently shown for some of them. In this report, we describe the structural and functional characterization of mouse S5D-SRCRB, a new group B member of the SRCR-SF. The s5d-srcrb gene maps at mouse chromosome 7 and encompasses 14 exons extending over 15 kb. The longest cDNA sequence found is 4286 bp in length and encodes a mature protein of 1371 aa, with a predicted molecular weight of 144.6 kDa. Using an episomal mammalian-expression system, a glycosylated soluble recombinant form >200 kDa was obtained and used as immunogen for the generation of specific rat mAbs. Subsequent immunohistochemical and real-time PCR analysis showed significant S5D-SRCRB expression in murine genitourinary and digestive tracts. S5D-SRCRB was shown to bind endogenous extracellular matrix proteins (laminin and galectin-1), as well as PAMPs present on Gram-positive and Gram-negative bacteria and fungi. PAMP binding by S5D-SRCRB induced microbial aggregation and subsequent inhibition of PAMP-induced cytokine release. These abilities suggest that S5D-SRCRB might play a role in the innate defense and homeostasis of certain specialized epithelial surfaces. The Journal of Immunology, 2011, 186: 2344–2354.
independently, giving rise to structurally and functionally different molecules, although with cooperative and complementary defensive and homeostatic functions. Examples of PRRs include proteins with leucine-rich repeats (i.e., TLRs), C-type lectin domains (i.e., dectin-1, DC-SIGN), and scavenger receptors (i.e., SR-A/I, CD36, MARCO).

The scavenger receptor cysteine-rich superfamily (SRCR-SF) is an ancient and highly conserved group of membrane-bound and/or soluble proteins mostly reported in the animal kingdom, from low invertebrates to mammals (3–5), as well as in some aquatic plants [i.e., unicellular green alga (6)]. In mammals, SRCR-SF members can be expressed by hemopoietic and nonhemopoietic cells, at embryonic and adult developmental stages, depending on species and tissue type. These proteins were reported to play a role in the regulation of innate and adaptive immune responses, as well as in the development of the immune system (3–5). Members of the SRCR-SF are characterized by the presence of one or several repeats of a highly conserved cysteine-rich extracellular scavenger receptor cysteine-rich (SRCR) domain (400–110 aa in size), which was first reported on the mouse macrophage scavenger receptor type I (SR-AI) (7). Depending on the number of cysteine residues present in the SRCR domain and the number of exons coding for each domain, the SRCR-SF can be divided into two mutually exclusive groups: A and B. Group A contains SRCR domains encoded by two or more exons and including six cysteines forming three disulphide bonds, whereas group B domains are encoded by a single exon and contain eight cysteines forming four disulphide bonds (3–5).

The extracellular regions of SRCR-SF members may present as exclusively composed of SRCR domains repeated in tandem or as multidomain mosaic proteins in which the SRCR domains seem to be combined with other types of protein modules, such as epitherial growth factor, C1r/C1s Uegf Bmp1, zona pellucida, collageneous regions, fibronectin, and short consensus repeats. The presence of short Pro, Ser, and Thr (PST)-rich polypeptides interspaced with contiguous SRCR domains is also frequently observed among SRCR-SF members. Available three-dimensional structures obtained from crystallization experiments indicate that group A and B SRCR domains present a highly conserved and compact core folding (a β sheet cradling an α helix) with variable outer loop regions, likely giving rise to functional diversity (8–13).

Despite the overall structure conservation of the SRCR domains across different species, no unifying biological function has been reported. They do not possess enzymatic activity; however, some SRCR domains have been involved in protein–protein interactions, the best studied examples being those of CD6 with CD166/ALCAM (4) and CD163 with the haptoglobin–hemoglobin complex (14). In recent years, a number of studies also supported the recognition of PAMPs by some, but not all, group A (i.e., MARCO) (15) and B (i.e., DMBTI/SAG/gp340, Spox, CD6, CD5, and CD163) (16–20) SRCR-SF members. Group B is composed of a dozen members expressed in mammals by immune cells, such as macrophages (i.e., CD163/M130, CD163L1/M160, Spox/AIM) or lymphocytes (i.e., CD5, CD6, SCART, WC1), as well as by cells of the gastrointestinal, respiratory, and genitourinary tracts (i.e., DMBT1/SAG/gp340, Spox/SCAT) or short consensus repeats. The presence of short Pro, Ser, and Thr (PST)-rich polypeptides interspaced with contiguous SRCR domains is also frequently observed among SRCR-SF members. Available three-dimensional structures obtained from crystallization experiments indicate that group A and B SRCR domains present a highly conserved and compact core folding (a β sheet cradling an α helix) with variable outer loop regions, likely giving rise to functional diversity (8–13).

Identification and genomic characterization of s5d-srcrb

Bioinformatic tools available in public databases (http://www.ncbi.nlm.nih.gov) were used to search for new members of group B of the SRCR-SF. The high-throughput genome sequences and the nonredundant database sections of GenBank (http://www.ncbi.nlm.nih.gov/Genbank) were screened using the BLASTN algorithm with the protein sequences of CD5 and CD6 as templates (Acc. No. X04391 and U34623, respectively). Mouse genomic clones with significant similarities (Evalue < 10–10) were selected and analyzed through the BLASTN algorithm against the mouse expressed sequences tags (ESTs) database of GenBank. The ESTs or full-length clones of interest were obtained from distribution centers already cloned into pFLCI and were further sequenced in both directions with the ABI PRISM dRhodamine terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Warrington, U.K.) using universal M13 primers (M13-21.0w 5’-GTAAAACGACGGCCAGT-3’). The AK079906 or A430110N23 full-length cDNA sequences (gi: 26348280) were used for mouse chromosome mapping of s5d-srcrb (http://www.ncbi.nlm.nih.gov/Mapview). Similarly, determination of exon/intron boundaries was done by sequencing with the mouse genomic sequence AC0794876 (gi: 9964851) using the BLAST2 tool (http://www.ncbi.nlm.nih.gov/BLAST).

Cloning and expression of s5d-srcrb

The full-length cDNA of s5d-srcrb tagged with a C-terminal hemagglutinin (HA) sequence was cloned into a modified version of the pCPE-Pu/AC7 mammalian expression vector (kindly provided by Takako Sasaki, Max-Planck-Institut für Biochemie, Martinsried, Germany). HEK 293 cells stably expressing TLR2 were a kind gift from Dr. Golenbock (University of Massachusetts Medical School, Worcester, MA). HEK 293-EBNA cells were grown in DMEM/F12 (Life Technologies Life Science, Grand Island, NY) supplemented with antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin), 10% heat-inactivated FCS (Walkersville, MD), and 250 µg/ml Geneticin (Life Technologies).

Materials and Methods

PBS (Roche Diagnostics, Mannheim, Germany) contained 137 mM NaCl, 3 mM KCl, 8 mM Na2HPO4, 1.5 mM KH2PO4 (pH 7.4). TBS contained 50 mM Tris-HCl (pH 7.4), 140 mM NaCl. Radioimmunoprecipitation assay (RIPA) buffer contained 50 mM Tris (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS supplemented with complete protease inhibitors (Roche Diagnostics), and 1 mM Na3VO4.

Cell lines

The human embryonic kidney cell line HEK 293-EBNA, which stably expresses the EBNA-1 Ag from EBV, was kindly provided by Takako Sasaki (Max-Planck-Institut für Biochemie, Martinsried, Germany). HEK 293 cells stably expressing TLR2 were a kind gift from Dr. Golenbock (University of Massachusetts Medical School, Worcester, MA). HEK 293-EBNA cells were grown in DMEM/F12 (Life Technologies Life Science, Grand Island, NY) supplemented with antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin), 10% heat-inactivated FCS (Walkersville, MD), and 250 µg/ml Geneticin (Life Technologies).
Lipofectamine 2000 reagent (Invitrogen, Paisley, U.K.), according to the manufacturer’s protocol. Transfectants were selected 48 h later in DMEM/ FCS/Geneticin plus 1 µg/ml Puromycin (Sigma, St. Louis, MO) and left to grow to confluence. To perform protein-expression analysis, confluent transfectants were cultured in serum-free DMEM/F12 medium (without selecting antibiotics), and supernatants were collected every 48–72 h; storage of supernatants was at −20°C after supplementation with 0.5 mM PMSF, 0.02% sodium azide, and 2.5 mM EDTA. Supernatant samples (1.5 ml) were precipitated with TCA and run over 6–8× SDS-PAGE under reducing conditions, followed by electrotransfer to nitrocellulose membranes (Bio-Rad Laboratories, Richmond, CA). For Western blot analysis, membranes were blocked for 1 h at room temperature with PBS containing 10% nonfat milk and then incubated with a mouse anti-HA mAb (cat. #sc-7392; Santa Cruz, Santa Cruz, CA, CA). After extensive washing with PBS 0.1% Tween 20, membranes were further incubated with a 1:1,000 dilution (in blocking buffer) of HRP-conjugated sheep anti-mouse IgG (Amersham Biosciences, Fairford, CT). Blots were visualized by chemiluminescence with Supersignal West Dura Extended Duration Solution (Pierce, Rockford, IL).

Real-time retrotranscription quantitative PCR assays

Analysis of the expression pattern of s5d-srcrb was performed in C57BL/6J mouse tissues by real-time retrotranscription quantitative PCR (RT-PCR) analysis. Total RNA was extracted using the TRizol reagent method, as recommended by the manufacturer (Invitrogen). cDNA synthesis was performed using the GeneAmp PCR kit (Roche). Briefly, 0.5–1 µg total RNA was mixed with 2.5 µl oligo(d-T)16 primer, 1 mM 2'-deoxyadenosine 5'-triphosphates, 1× PCR Buffer II, and 5 mM MgCl₂. After denaturing for 5 min at 65°C, 20 U RNase inhibitor and 50 U Moloney murine leukemia virus reverse transcriptase were added (total reaction volume, 20 µl). The reaction was incubated for 90 min at 42°C and stopped for 15 min at 99°C. cDNA integrity was checked by PCR using primers specific for the housekeeping 18S gene.

Bacterial and fungal protein-binding assays
E. coli ATCC25922 and Staphylococcus aureus ATCC29213 were obtained from the American Type Culture Collection. Schizosaccharomyces pombe and Saccharomyces cerevisiae were kindly provided by the Cell Biology Unit of the University of Barcelona. The rest of the bacterial and fungal strains used in this study are clinical isolates characterized by the Department of Microbiology, Hospital Clinic of Barcelona, using standard biochemical procedures. Bacteria were grown overnight at 37°C in Luria-Bertani medium, and fungi were grown at 28°C in Sabouraud’s medium with aeration, harvested by centrifugation at 1500 rpm for 10 min, and resuspended in TBS to a final density of 10⁸ bacteria/ml or 10⁸ fungi/ml. Antibacterial and antifungal assays were performed in triplicate. For bacterial and fungal suspensions, microbial-binding assays, aliquots with a final density of 10¹⁰ bacteria/ml or 10⁸ fungi/ml were added to the bacterial/fungal suspensions to a final concentration of 50 µM and then added to the bacterial/fungal suspensions to a final concentration of 10¹⁰ bacteria/ml or 10⁸ fungi/ml. After incubation with serum-free rmS5D-SRCRB-HA supernatant for 1 h at 4°C under gentle orbital rotation, the beads were washed again, before, and treated with different glycosidase combinations from the Enzymatic Protein Deglycosylation Kit (Sigma), following the manufacturer’s instructions. The resulting products were resolved by SDS-PAGE and analyzed by Western blot, as described above.

Western blot detection of mouse S5D-SRCRB in cell transfectants and tissues

Tissue samples from C57BL/6J males were snap-frozen in liquid nitrogen and then weighed, minced, and mixed with three volumes of RIPA lysis buffer for homogenization with a Teflon-glass Dounce, followed by sonication with two short bursts (2 s) in a Barnson Sonifier 250 (Danbury, CT). Tissues were kept on ice for 20 s in between bursts. After clarification at 12,000 rpm for 20 min at 4°C, total protein content from supernatants was quantified by the Bradford method (BCA Protein assay kit; Pierce), as recommended by the manufacturer. For control purposes, 1× 10⁶ HEK 293-EBNA transfectants expressing rmS5D-SRCRB-HA were lysed following the same procedure. Protein samples (100 µg) were resolved by 6–7.5% SDS-PAGE and then electrotransferred to a nitrocellulose membrane (BioRad, Durham, NC). Subsequently, Western blot analysis was performed using a 1:2 dilution of rat 1H11.A8.G2 hybridoma supernatant plus goat HRP-conjugated anti-rat IgG (Sigma), as previously described.

Deglycosylation analyses

The rmS5D-SRCRB-HA was immunoprecipitated using serum-free rat anti–S5D-SRCRB 1H11.A8.G2 hybridoma supernatant previously adsorbed for 1 h at 4°C under orbital rotation with 50 µl a 50% (v/v) suspension (in PBS) of Protein G Sepharose Fast Flow (GE Healthcare, Upssala, Sweden). The beads were pulled down, washed three times with PBS, and incubated with serum-free rmS5D-SRCRB-HA supernatant for 1 h at 4°C under gentle rotation. The beads were washed again, before, and treated with different glycosidase combinations from the Enzymatic Protein Deglycosylation Kit (Sigma), following the manufacturer’s instructions. The resulting products were resolved by SDS-PAGE and analyzed by Western blot, as described above.

Generation of rat anti–SSD-SRCRB mAbs

For immunization purposes, each Sprague-Dawley rat was injected i.p. fortnightly with TCA precipitates from 12 ml FCS-free supernatant samples of recombinant HA-tagged mouse SSD-SRCRB (rm-S5D-SRCRB-HA)–producing stable HEK 293-EBNA transfectants. In brief, FCS-free supernatant was precipitated with a 1:10,000 dilution of TCA precipitates from 12 ml FCS-free supernatant samples was examined by fluorescence microscopy. The plasmid used for bacterial expression of GST-Galectin 1 (GST-Gal1) strains used in this study are clinical isolates characterized by the Department of Microbiology, Hospital Clinic of Barcelona, using standard biochemical procedures. Bacteria were grown overnight at 37°C in Luria-Bertani medium, and fungi were grown at 28°C in Sabouraud’s medium with aeration, harvested by centrifugation at 1500 rpm for 10 min, and resuspended in TBS to a final density of 10¹⁰ bacteria/ml or 10⁸ fungi/ml. After centrifugation at 3500 rpm for 10 min, bacterial and fungal suspensions were added to 1:20 diluted rat 1H11.A8.G2 hybridoma supernatant plus anti–S5D-SRCRB 1H11.A8.G2 hybridoma supernatant previously adsorbed for 1 h at 4°C under orbital rotation with 50 µl a 50% (v/v) suspension (in PBS) of Protein G Sepharose Fast Flow (GE Healthcare, Upssala, Sweden). The beads were pulled down, washed three times with PBS, and incubated with serum-free rmS5D-SRCRB-HA supernatant for 1 h at 4°C under gentle rotation. The beads were washed again, before, and treated with different glycosidase combinations from the Enzymatic Protein Deglycosylation Kit (Sigma), following the manufacturer’s instructions. The resulting products were resolved by SDS-PAGE and analyzed by Western blot, as described above.

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mM) for an additional 4 h. The bacterial cell pellet was subjected to three cycles of freezing/thawing and was sonicated and solubilized with 10% Triton X-100 in the presence of protease inhibitors (Complete; Roche). The clarified supernatant was mixed with Glutathione Sepharose 4B beads (GE Healthcare) for 2 h at 4˚C under gentle orbital rotation. The beads were then washed thoroughly with TBS 1% Triton X-100, resuspended in TBS, and kept at 4˚C until used. GST-1–Sepharose beads were incubated for 90 min at 4˚C in blocking buffer (TBS plus 5% BSA). Then, 10 μl serum-free media (24 h) were added to each well and incubated for 2 h bound at room temperature. Bound protein was detected by a 1-h incubation at room temperature with neat serum-free 1H11.A8.G2 rat hybridoma supernatant, followed by a 30-min incubation with a 1:1000 dilution of goat anti-rat IgG/HRP antiserum (Sigma). Between each incubation step, excess unbound proteins were washed off three times with PBS 0.01% Tween 20. Color was developed by adding 3,3’-5,5’-tetramethylbenzidine (TMB) liquid substrate (Sigma), and the absorbance was measured at 450 nm. The assay was repeated at least three times with similar results.

Interaction of rm5D5-SRCRB-HA with PAMP was analyzed by coating 96-well microtiter plates (Nunc, Roskilde, Denmark) overnight at 4˚C with 2 μg/well purified LPS from E. coli K12 (InvivoGen, San Diego, CA), PGN (cat. #77140; Sigma), LTA from well purified LPS from E. coli K12 (InvivoGen, San Diego, CA), or TBS alone to remove unbound protein. The proteins were eluted with 0.02% SDS-PAGE under reducing conditions for further Western blot analysis. Laemmli’s sample buffer and separated on SDS-PAGE under reducing conditions for further Western blot analysis. Beads were washed twice with TBS 0.01% Tween 20 and once with TBS alone to remove unbound protein. The proteins were eluted with

ELISA assays

Interaction of rm5D5-SRCRB-HA with PAMP was analyzed by coating 96-well microtiter plates overnight at 4˚C with 2 μg/well purified LPS from E. coli K12 (InvivoGen, San Diego, CA), PGN (cat. #77140; Sigma), LTA from S. aureus (cat. #2515; Sigma), Zymosan A (cat. #2450; Sigma), glucan from bakers’ yeast (cat. #5011; Sigma), β(1–3)-glucan (cat. #89662; Sigma), β-D-glucan (cat. #6513; Sigma), or mannan (cat. #m7504; Sigma) in coating buffer (100 mM NaHCO₃ [pH 9.5]). Non-specific binding to plastic wells was prevented by incubation for 1 h at room temperature in blocking solution (PBS plus 3% BSA). Serial 2-fold dilutions of serum-free rm5D5-SRCRB-HA supernatants were added to the wells and incubated for 2 h bound at room temperature. Bound protein was detected by a 1-h incubation at room temperature with neat serum-free 1H11.A8.G2 rat hybridoma supernatant, followed by a 30-min incubation with a 1:1000 dilution of goat anti-rat IgG/HRP antiserum (Sigma). Between each incubation step, excess unbound proteins were washed off three times with PBS 0.01% Tween 20. Color was developed by adding 3,3’-5,5’-tetramethylbenzidine (TMB) liquid substrate (Sigma), and the absorbance was measured at 450 nm. The assay was repeated twice with similar results.

Immuno histochemical assays

Four-micrometer sections from formaldehyde-fixed paraffin-embedded tissue blocks were mounted on ChemMate Capillary Gap Slides (Dako, Glostrup, Denmark), dried at 60˚C, deparaffinized, and hydrated. Prior to Ag retrieval, blocking of endogenous peroxidase was performed in 1.5% hydrogen peroxide in TBS buffer (pH 7.4) for 10 min. Ag retrieval was carried out using microwave heating in target retrieval solution (Dako) or pepsin. Three Tissue-Tek containers (Miles, Elkhart, IN), each with 24 slides in 250 mL buffer, were placed on the edge of a turntable inside the microwave oven. Slides were heated for 11 min at full power (900 W) and then for 15 min at 400 W. After heating, slides were left in buffer for 15 min. Incubation with 1:10 diluted supernatants (in Ab Diluent S2022; Dako) from anti–5D5-SRCRB hybridomas (H111.A2.G8, 4D11.A2.H4, 8F4.F3.F9, S5F4.F2, 8B2.H4, 1C11.D6.C9, 74G1.E1.H7, and 5E12.G3.B1) was carried out for 60 min at room temperature. No immunostaining was seen when the primary Ab was omitted or substituted with an isotype control. Immunostaining was automated using the PowerVision+ HRP detection system DPVB+500HRP (ImmunoVision Technologies, Brisbane, CA) on the TechMate 500 instrument (Dako). DAB+ (K3468; Dako) was used as substrate-chromogen system. Immunostaining was followed by brief nuclear counterstaining with Mayer’s hematoxylin. Finally, cover slips were mounted with AquaTex (Merck, Darmstadt, Germany).

IL-8–release assay

HEK 293 cells and HEK 293 cells stably expressing TLR2 were plated on a 96-well plate at a density of 5 × 10⁴ cells/well for 24 h and then cultured in serum-free media for an additional 24 h. Cells were then pulsed with 20 μg/ml PGN for 24 h in the presence of supernatants from HEK 293-EBNA transfectants expressing rm5D5-SRCRB-HA or from untransfected cells as a control. Culture supernatant samples (25 μl) were collected and assayed for human IL-8 by ELISA (BD OptEIA, Human IL-8 ELISA Set; BD Biosciences, San Diego, CA), following the manufacturer’s instructions.

Results

Identification and cloning of mouse s5d-srcrb

The screening of GenBank databases with the CD5 and CD6 protein sequences rendered two Mus musculus P1-derived artificial chromosome genomic clones (Acc. No. AC079486 and AC079542) predicted to contain a gene coding for a protein containing several group B SRCR domains. These predicted genes contained the same similarities (Evalue < 10⁻⁸) to PAC genomic clones, 17 of which showed the highest score (Supplemental Table I) and were expressed in different tissues (muscle, brain, mammary gland, pancreas, liver, eye, gastrointestinal tract) and at different developmental stages (from embryo to adult). Two 4286 bp-long cDNA clones (Acc. No: AK079906 and A430110-N23) obtained from thymus tissue of C57BL/6 mice at day 0 of neonatal development were cloned into the pFLCI vector to be fully resequenced. They were shown to contain an open reading frame of 4116 bp encoding a polypeptide chain of 1371 aa (Acc. No: BAC37780 and NP_766596), with a predicted Mᵣ of 144.6 kDa (Supplemental Fig. 1). This putative new protein (Acc. No: EU850434, gi: 194354452) contained a short leader peptide and five SRCR domains (each was ~100 aa in size) (http://www.ncbi.nlm.nih.gov/structure/cdd). The N-terminal signal peptide sequence was 16 aa long, and the most likely signal peptide cleavage site was IQA–IE (Signal IP 3.0; http://www.cbs.dtu.dk). Four Asn-X-Ser/Thr motifs were found, three of which (NETA, NSTA, and NTTS) are predicted to be N-glycosylated (NetNGlyc 1.0; http://www.cbs.dtu.dk). All five SRCR domains fulfilled the characteristics of group B (encoded by a single exon and containing eight properly spaced cysteines) (5) and were interspersed by short (between SRCR1–SRCR2, and SRCR3–SRCR4), intermediate (between SRCR4–SRCR5), or long (C-terminal to SRCR5) PST-rich regions. Multiple O-glycosylation sites were predicted, which were mainly located within the PST-rich sequences (NetOGlyc 3.1; http://www.cbs.dtu.dk) (Fig. 1A, 1B). The PST-rich region C-terminal to SRCR5 was predicted to contain a 155-aa-long sequence (from aa 1050–1205) with homology to syndecan domains (http://www.ncbi.nlm.nih.gov/structure/cdd). No transmembrane region could be predicted (TMHMM; http://www.cbs.dtu.dk), as would be expected for a secreted protein. The mouse gene encoding this cDNA sequence was named s5d-srcrb, for soluble protein with 5 domains of the SRCR group B, and was mapped to chromosome 7 (http://www.ncbi.nlm.nih.gov/mapview). It spans 15 kb and encompasses 14 exons (Fig. 1A). Exons 1 and 2 encode the 5′-untranslated region and the signal peptide. Exons 3, 6, 7, 9, and 11 encode each of the five SRCR domains as expected for group B members. Exons 4, 5, 8, and 10 encode the short and intermediate PST-rich intervening regions. Exons 12–14 encode the long PST-rich C-terminal region (encompassing the putative syndecan domain), the stop codon, and the 3′-untranslated region with the polyadenylation signal sequence (AAGAAA) at 14 bp upstream from the poly(A) tail.

Through the same bioinformatic tools used to identify s5d-srcrb, a homologous gene mapping to an orthologous region of human chromosome 19 was also identified. It corresponds to a recently reported human gene (SSc5D), for which no biochemical and functional data are available (22). Multiple alignments of the amino acid sequences of the SRCR domains from the human and mouse genes show a high degree (~60%) of interdomain, as well as interspecies amino acid identity (Fig. 1C). This indicates that the five SRCR domains may have arisen from exon duplication.
Expression of rmSSD-SRCRB-HA and generation of an SSD-SRCRB–specific rat mAb

The full-length cDNA sequence of mouse s5d-srcrb was fused in frame with a C-terminal HA tag and then cloned into the pCEP-Pu vector for further expression into HEK 293-EBNA cells. Serum-free supernatants from stable transfecants expressing this rmSSD-SRCRB-HA protein were precipitated using TCA, separated on SDS-PAGE under reducing conditions, and subjected to Western blot with an anti-HA mAb. This analysis rendered a single broad band of ∼200 kDa in size (Fig. 2A), which is far greater than the predicted Mr (144.6 kDa) for the s5d-srcrb gene product. The difference between the observed and expected Mr argues in favor of rmSSD-SRCRB-HA undergoing posttranslational modifications. This was further confirmed by simultaneous Western blot analysis of cell solubilizes and culture supernatants from HEK 293-EBNA transfecants expressing rmSSD-SRCRB-HA. As seen in Fig. 2C, the intracellular form of rmSSD-SRCRB-HA was shown to display a smaller Mr (∼150 kDa) than did the extracellular one (∼200 kDa).

The rmSSD-SRCRB-HA present in serum-free supernatants from stable transfecants was used as immunogen to generate specific rat mAbs. After several cloning and subcloning steps, seven rat hybridomas were selected (Supplemental Table II). Their specificity was initially analyzed using ELISA plates precoated with serum-free rmSSD-SRCRB-HA–containing supernatants. None of the selected hybridomas was reactive to other HA-tagged group B SRCR-SF members’ protein, such as S4D-SRCRB (24). The same results were obtained following immuno precipitation and Western blot analyses of rmSSD-SRCRB-HA supernatant samples, further indicating that all rat mAbs generated recognized rmSSD-SRCRB-HA under native (ELISA, immunoprecipitation) and denaturing (Western blot) conditions; this suggests that the mAbs are likely recognizing linear epitopes.

Glycosylation studies

Because the predicted amino acid sequence of mouse S5D-SRCRB contains multiple N- and O-linked glycosylation sites, the possibility that glycosylation could account for the above-mentioned posttranslational modifications was assessed. rmSSD-SRCRB-HA was immunoprecipitated and then subjected to incubation with different glycosidases, either alone or in combination. As illustrated in Fig. 2B, little or no change in Mr was observed when rmSSD-SRCRB-HA was incubated with galactosidase, which releases only β(1→4) terminal galactose residues. Partial sensitivity could be observed to N-acetylgalactosaminidase (Fig. 2B) and PNGase F (data not shown), as deduced from the generation of a faint band ∼150 kDa, which is the predicted Mr for the unprocessed intracellular form of S5D-SRCRB. Because N-acetylgalactosaminidase cleaves all terminal β-linked N-acetylglucosamine residues typical of N-linked glycosylations, and PNGase F cleaves all types of Asn-linked sugars, some degree of N-glycosylation can be inferred for S5D-SRCRB, which is in agreement with the presence of putative N-glycosylation sites. Combined digestion with neuraminidase and O-glycosidase induced a small reduction in the observed Mr of rmSSD-SRCRB-HA (Fig. 2B), which was similar to that observed for neuraminidase or O-glycosidase alone (data not shown). This indicates that S5D-SRCRB also undergoes some degree of O-glycosylation, as predicted from its relatively high content of PST-rich sequences. In parallel experiments, all of the glycosidases gave optimal digestion results when bovine fetuin was used as a control glycoprotein (data not shown). These results indicated that, although likely glycosylated, rmSSD-SRCRB-HA is also relatively resistant to glycosidase treatment, as has often been reported for some heavily glycosylated high Mr proteins (25, 26).

Tissue-expression analysis of s5d-srcrb

The expression pattern of S5D-SRCRB in normal mouse tissues was assessed by immunohistochemistry (IHC) and RT-qPCR assays. Of the seven rat hybridomas generated, two (4D11.A2.H4 and 1H11.A8.G2) were shown to be appropriate for IHC studies on paraffin-embedded tissues. The two mAbs gave identical staining results with different retrieval methods (pepsin or target retrieval solution). As illustrated in Fig. 3A, positive immunostaining was detected throughout the gastrointestinal and genitourinary tracts. Strong staining of serosal salivary gland and the exocrine part of pancreas, as well as of testis, was observed. In kidney, selective

FIGURE 1. Gene and protein domain organization of mouse s5d-srcrb. A, the exon–intron organization of mouse s5d-srcrb and its correlation with the domain organization of the predicted cDNA. B, location of putative O-glycosylation sites along the protein sequence, according to the NetOGlyc 3.1 software. C, alignment of amino acid sequences of SRCR domains from the predicted mouse and human S5D-SRCRB proteins. Conserved cysteine residues are shown in black boxes. The gray boxes correspond to regions with >80% amino acid conservation.

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of lower Mr, (~80 kDa) was also repeatedly observed in testis, likely representing an alternatively spliced isoform or a partially degraded form. Several attempts to immunoprecipitate SSD-SRCRB from testis and other positive tissues (pancreas, kidney) were unsuccessful. This indicated that the epitopes recognized by the anti-SSD-SRCRB mAbs are inaccessible in the endogenous protein under the solubilization conditions used (RIPA buffer) or, more likely, that the mAbs do not bind properly in the stringent solubilization buffer conditions used during RIPA-immunoprecipitation.

We also set out to study whether the expression of mouse SSD-SRCRB is regulated during embryo development. Preliminary in situ RNA-hybridization analysis carried out at day 9.5 postcoitum showed that SSD-SRCRB is selectively detected in placodes, embryonic ectodermal thickenings where organs or structures will develop (Supplemental Fig. 3). RT-qPCR experiments also showed a substantial increase in SSD-SRCRB RNA expression from days 9–14 postcoitum (Supplemental Fig. 4). These data suggested that the expression of SSD-SRCRB is developmentally regulated.

Pathogen-binding and aggregating properties of recombinant SSD-SRCRB

Given that some members of the SRCR-SF act as receptors for PAMPs, the microbial-binding properties of rmSSD-SRCRB-HA were assessed in vitro. Bacterial or fungal cell suspensions were incubated with serum-free rmSSD-SRCRB-HA–containing supernatants, and the presence of cell-bound protein was tested by Western blot. As shown in Fig. 4A (left panel), rmSSD-SRCRB-HA could be detected in cell pellets from most bacterial strains assayed, either Gram-negative (E. coli, Salmonella typhimurium, Yersinia enterocolitica, Shigella flexneri) or Gram-positive (S. aureus, Staphylococcus epidermidis). Relatively low binding signal was observed for A. baumannii. In parallel assays, rmSSD-SRCRB-HA also bound to all of the fungal strains analyzed, either saprophytic (S. cerevisiae, S. pombe) or pathogenic (C. albicans, Cryptococcus neoformans, A. fumigatus) (Fig. 4A, right panel). In all cases tested, the binding of rmSSD-SRCRB-HA was dose dependent (Fig. 4B). In full agreement with its pathogen-binding properties, rmSSD-SRCRB-HA was also able to induce bacterial and fungal aggregation, an effective mechanism to avoid dissemination of pathogenic agents and, thus, to control infection. As shown in Fig. 5, addition of rmSSD-SRCRB-HA, but not BSA, to microbial suspensions induced aggregation of Gram-positive (S. aureus) and Gram-negative (E. coli, A. baumannii) bacteria, as well as fungi (C. albicans).

PAMP recognition by SSD-SRCRB

The nature of the cell wall component(s) responsible for the bacterial- and fungal-binding ability of mouse SSD-SRCRB was studied by ELISA. Plastic plates were coated with different PAMPs (PGN, LPS, LTA, zymosan, glucan, mannan) and then incubated with serum-free rmSSD-SRCRB-HA supernatants. The presence of bound mSSD-SRCRB-HA was detected using either anti-SSD-SRCRB rat mAb plus HRP-conjugated anti-rat IgG antiserum. As shown in Fig. 6, significant dose-dependent binding of rmSSD-SRCRB-HA could be detected to PGN, LPS, zymosan, and linear β-glucan but not to LTA, mannan, or branched β-glucans.

Binding of testis SSD-SRCRB proteins to microbial surfaces

Next, we also studied whether the endogenous mouse SSD-SRCRB protein form(s) expressed in testis also retain(s) the microbial-binding properties of the recombinant protein (rmSSD-SRCRB-HA). S. aureus was chosen for these studies because our results showed that it gave the best binding. Thus, bacterial cell sus-

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**FIGURE 2.** Analysis of recombinant and tissue-expressed mouse SSD-SRCRB. A, Expression of rmSSD-SRCRB-HA by human HEK 293-EBNA cells. Samples of TCA-precipitated serum-free culture supernatants from transfected (+) and untransfected (−) cells were run on 7.5% SDS-PAGE under reducing conditions and transferred to nitrocellulose for Western blot analysis with an anti-HA mAb plus HRP-conjugated anti-mouse IgG antiserum. B, Glycosylation analysis of rmSSD-SRCRB-HA. Serum-free culture supernatants from HEK 293-EBNA transfectants expressing rmSSD-SRCRB-HA were immunoprecipitated with 1H11.A8.G2 mAb. The immune complexes were then incubated for 3 h at 37˚C with the indicated glycosidases, alone (galactosidase, N-acetylgalactosaminidase) or in combination (neuraminidase plus O-glycosidase [Nase+O-Gly]). The resulting products were analyzed by Western blot using a 1:2 dilution of rat 1H11.A8.G2 hybridoma supernatant plus goat HRP-conjugated goat antimouse IgG. C, Comparative Western blot analysis of recombinant and tissue-expressed mouse SSD-SRCRB. Samples of serum-free culture supernatants (extracel.) and detergent (1% Nonidet P-40) cell solubilates (intracel.) from stable HEK 293-EBNA transfectants were analyzed in parallel with soluble fractions of RIPA homogenates from mouse heart and testis. Samples were run on 7.5% SDS-PAGE under reducing conditions and transferred to nitrocellulose for Western blot analysis, as in B.
Pensions were incubated with liver and testis tissue solubilizates and then pelleted, extensively washed, and resolved by SDS-PAGE under reducing conditions for further Western blot analysis. As shown in Fig. 4C, anti–S5D-SRCRB–reactive bands were recovered in *S. aureus* cell pellets adsorbed onto testis but not liver homogenates. The observed bands corresponded to the greatest (fully processed) and smallest (alternatively spliced or proteolytically processed) Mr forms of mouse S5D-SRCRB (Fig. 2C). No evidence of reactivity with the intermediate Mr (intracellular, incompletely processed) form was observed.

**Inhibition of PAMP-induced cytokine release by S5D-SRCRB**

Our results showed that rmS5D-SRCRB-HA bound to bacteria and was able to induce aggregation. We next studied whether rmS5D-SRCRB-HA has intrinsic bactericidal activity. As shown in Supplemental Fig. 5, our preliminary results suggested that rmS5D-SRCRB-HA does not display measurable bactericidal activity at a concentration of 1 μg/ml. Similar results were obtained with rmS5D-SRCRB-HA concentrations ≤20 μg/ml (data not shown).

We then sought to determine whether the interaction of S5D-SRCRB with PAMP could affect a biological outcome, such as cytokine release. HEK 293 cells stably transfected with TLR2 release IL-8 when exposed to PGN, a known PAMP and TLR2 ligand. We then exposed these cells, as well as untransfected HEK 293 cells, to PGN in the presence or absence of rmS5D-SRCRB-HA–containing supernatant. As shown in Fig. 7, release of IL-8 induced by PGN was dose-dependently inhibited by rmS5D-SRCRB-HA; inhibition was significant (*p* < 0.05) when 100 μl supernatant was used. Supernatant from untransfected HEK 293 cells did not affect IL-8 release by HEK 293 TLR2 cells (data not shown).

**Binding of mouse SSD-SRCRB endogenous extracellular proteins**

Archetypical members of the SRCR-SF (e.g., DMBT1/gp340/SAG) were reported to interact with exogenous pathogens, as well as with endogenous host components (27). Therefore, it was tested whether this could also be the case for S5D-SRCRB. First, we explored its binding to different purified extracellular matrix proteins by ELISA. As illustrated by the results presented in Fig. 8A, rmS5D-SRCRB-HA showed clear dose-dependent binding to laminin. Low (fibronectin) or negative (collagen I and IV) binding was observed for other extracellular matrix components. An additional experiment demonstrated the sugar-dependent interaction of S5D-SRCRB with galectin-1, a broadly expressed homodimeric mammalian lectin secreted by epithelial cells (28). As shown in Fig. 8B, specific binding of rmS5D-SRCRB-HA to GST-Gal1 Sepharose beads was competed in a dose-dependent manner.
short, although still-growing, list of members of the SRCR-SF showing pathogen-binding properties. This ability has been unequivocally mapped to the SRCR itself in only a few members, including MARCO, CD163, DMBT1/SAG/gp340, CD5, CD6, and

![FIGURE 4. Binding of mouse SSD-SRCRB to microbial organisms. A. Direct binding assays of rmSSD-SRCRB-HA to bacterial and fungal strains. Samples (250 µl) of serum-free supernatants from stable transfectants expressing rmSSD-SRCRB-HA were incubated for 1 h at 4°C with the indicated bacterial (5 × 10^7) or fungal (5 × 10^6) cell suspensions in binding buffer (TBS, 1% BSA, 5 mM CaCl_2). Cell pellets were washed thoroughly and then resuspended in Laemmli’s sample buffer for further separation in 6% SDS-PAGE. Cell-bound protein was subsequently detected by Western blot analysis, as described in Materials and Methods. B. Dose-dependent binding of rmSSD-SRCRB-HA to bacterial and fungal strains. Increasing amounts of serum-free supernatants from stable transfectants expressing rmSSD-SRCRB-HA were incubated with 5 × 10^7 Gram-positive (S. aureus) and Gram-negative (E. coli) bacteria, as well as with 5 × 10^6 pathogenic (C. albicans) and saprophytic (S. cerevisiae) fungi. Bound protein was recovered and detected as described above. C. Binding of bacteria to tissue-expressed mouse SSD-SRCRB. Mouse testis and liver tissue was homogenized in three volumes of binding buffer (see above), and 100 µl of their soluble fractions was incubated for 1 h at 4°C with 5 × 10^7 S. aureus. Presence of cell-bound mouse SSD-SRCRB protein was analyzed by Western blot, as in A.

![FIGURE 5. Aggregation of bacterial and fungal cell suspensions by mouse SSD-SRCRB. The indicated strains of bacteria and fungi were labeled with FITC and incubated overnight at room temperature with 20 µg/ml of affinity-purified rmSSD-SRCRB-HA or BSA, used as negative control. After extensive washing, the cells were observed by fluorescence microscopy. Original magnification ×100.

![FIGURE 6. Binding of mouse SSD-SRCRB to conserved microbial cell wall components. A. Binding of rmSSD-SRCRB-HA to bacterial cell wall components. ELISA plates were coated overnight at 4°C with 2 µg/well of LPS, PGN, or LTA. After blocking with PBS 3% BSA, 2-fold serial dilutions of serum-free supernatants (100 µl) from transfectants expressing HA-tagged mouse SSD-SRCRB were added to wells and incubated for 2 h at room temperature. Bound protein was detected using 1H11.A8.G2 mAb plus HRP-conjugated goat anti-rat IgG antiserum. Plates were developed with TMB substrate solution, and additional absorbance was measured at 450 nm. B. Binding of rmSSD-SRCRB-HA to fungal cell wall components. ELISA plates were coated overnight at 4°C with 2 µg/well of zymosan, β-glucans (linear or branched), and mannan. Bound protein was detected using 1H11.A8.G2 mAb plus HRP-conjugated goat anti-rat IgG antiserum. Plates were developed with TMB substrate solution, and additional absorbance was measured at 450 nm.

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Discussion

Epithelial cells form physical barriers that are covered by mucosal surfaces aimed at preventing the entry of invading pathogens. Moreover, spontaneously or following the sensing of microbial components, epithelial cells can also synthesize effector molecules that trigger or increase the defensive immune responses of the host (i.e., mucosal inflammation and other related innate and adaptive immune responses) (29–33). The present work reports the identification and further molecular and functional characterization of a new mouse receptor (SSD-SRCRB) mainly expressed by some epithelial cell types and likely belonging to the humoral arm of the innate immune system. This receptor possesses the characteristics of group B members of the SRCR-SF (3, 5), and it should be considered the mouse homolog of SSc5D (22). A relevant finding of the current study is the demonstration that mouse SSD-SRCRB behaves as a PRR. Thus, SSD-SRCRB should be added to the

(3–30 mM) manner by lactose but not by another irrelevant sugar (sucrose). Taken together, the results indicated that, once secreted, SSD-SRCRB could be immobilized to relevant constituents of the extracellular milieu (e.g., laminin and galectin-1, and perhaps other unidentified proteins as well), thus putatively contributing to basic homeostatic epithelial cell functions.
Sp∞. In some instances, the amino acid sequence motifs involved in SRCR-mediated pathogen recognition have been precisely identified (RxR, VEVLxxxxW) (15, 16, 20), but an exact match to these motifs is not observed among any of the SRCR domains of SSD-SRCRB. However, by no means does this exclude the SRCR domains of mouse SSD-SRCRB as being responsible for pathogen recognition, as exemplified by Sp∞, CD6, and CD5 (17–19). The SRCR domains are among the few protein modules from which evolution has settled a myriad of structurally related but functionally different proteins. Key residues that stabilize the core structure of SRCR domains are well conserved, whereas amino acid positions at externally oriented connecting loops are keen to change, thus generating protein versatility. This versatility may have given rise to a certain degree of functional diversity among the members of the SRCR-SF that would justify the lack of a unifying function for all SRCR-SF members; this is exemplified even when exploring the pathogen-binding properties of SRCR-SF members. However, mutational investigations to exclude the possibility that the non-SRCR domains of SSD-SRCRB could account for its pathogen-binding properties remain to be performed.

Intriguingly, mouse SSD-SRCRB presents several structural and functional analogies with the human secreted protein DMBT1, also known as gp340 or salivary agglutinin (SAG), and which also corresponds to rat ebnerin, mouse Crp-ductin, or rabbit hensin (5). DMBT1/gp340/SAG is an archetypal group B SRCR-SF protein involved in infection, inflammation, and cancer (34). From a structural point of view, it is a mosaic-type glycoprotein composed of 14 SRCR domains interspersed by PST-rich sequences and possessing two C1r/C1s Uegf Bmpl and one zona pellucida domains at its C-terminal region. Functionally, DMBT1/gp340/SAG represents an innate defense factor interacting with a broad spectrum of pathogens (bacteria, fungi, viruses), as well as mucosal defense proteins (galectins, IgA, surfactant A and D proteins, MUC5B, TFF2). In contrast, it drives epithelial and stem cell differentiation as an extracellular matrix protein, whereas members. However, some of the functional analogies with the human secreted protein DMBT1, also known as gp340 or salivary agglutinin (SAG), which also corresponds to rat ebnerin, mouse Crp-ductin, or rabbit hensin (5). DMBT1/gp340/SAG is an archetypal group B SRCR-SF protein involved in infection, inflammation, and cancer (34). From a structural point of view, it is a mosaic-type glycoprotein composed of 14 SRCR domains interspersed by PST-rich sequences and possessing two C1r/C1s Uegf Bmpl and one zona pellucida domains at its C-terminal region. Functionally, DMBT1/gp340/SAG represents an innate defense factor interacting with a broad spectrum of pathogens (bacteria, fungi, viruses), as well as mucosal defense proteins (galectins, IgA, surfactant A and D proteins, MUC5B, TFF2). In contrast, it drives epithelial and stem cell differentiation as an extracellular matrix protein, whereas mouse SSD-SRCRB should also be considered a mosaic protein because it is composed of five SRCR domains interspersed by PST-rich sequences and possessing a C-terminal syndecan-like domain. Syndecans are heparan sulfate proteoglycans that are known to mediate interactions with extracellular matrix proteins and heparin-binding growth factors. Preliminary data support the putative interaction of rmSSD-SRCRB-HA with some extracellular matrix proteins, such as laminin and, to a lesser extent, fibronectin, but not collagen I or IV. Moreover, we also provide evidence on the carbohydrate-dependent interaction of mSSD-SRCRB-HA with galectin-1, a host soluble lectin that functions as a damage-associated molecular pattern and a receptor for PAMPs (35). The mucosal-defense properties of mouse SSD-SRCRB against living organisms are supported by its broad binding and aggregating microbial spectrum that includes saprophytic and pathogenic bacteria and fungi; potential interactions with viruses remain to be studied. Our preliminary data indicated that this defensive role is played in the absence of detectable intrinsic bactericidal activity, which is not unusual for innate-immunity proteins; SAG is a well-known example of a nonbacterial protein with defensive properties. By binding to microbial components, mouse SSD-SRCRB seems to downregulate subsequent PAMP-induced cytokine release, as deduced from our experiments showing dose-dependent inhibition of IL-8 release following PGN stimulation of HEK 293 TLR2 transfectants. We consider that this could be important for preserving the integrity and the function of epithelia from excessive or prolonged inflammation caused by PAMPs in those tissues where SSD-SRCRB is expressed. This is best exemplified by the expression of mouse SSD-SRCRB in seminiferous testicular tubules, where infection and inflammation may cause male infertility, thus compromising murine reproductive capability (36). The expression of mouse SSD-SRCRB in these tissues also argues in favor of its putative
role in cell-differentiation processes. Interestingly, preliminary data from our group indicate that expression of mouse SSSRCRB is regulated during embryo development. More precisely, in situ RNA hybridization results at day 9.5 postcoitum showed that SSSRCRB is selectively distributed in placodes, embryonic ectodermal thickenings where organs or structures will develop (37). Moreover, RT-qPCR data indicate that there is an important relative increase in its RNA expression from days 9–14 postcoitum, the meaning of which remains to be explored further. Finally, the relative preferential expression of mouse SSSRCRB observed in adult tissues also opens the possibility that it may behave as a nonchromosome X-encoded cancer/testis Ag (38). Therefore, a systematic investigation of mouse SSSRCRB expression in human and mouse tumors should be performed to exclude this possibility.

The very limited information available on human SScS5D highlights the need for future studies to explore whether the functional and structural characteristics reported in this article for mouse SSSRCRB also apply to its human homolog. At present, marked differences exist regarding their tissue-expression pattern. Although the highest expression level of SScS5D is reported in placental, spleen, colon, and lung, mouse SSSRCRB is mainly expressed in testis, kidney, and the serosal region of salivary and pancreas glands. To further clarify this point, parallel IHC analyses of mouse and human tissues performed with the 1H11.A8.G2 mAb, which shows human–mouse species cross-reactivity (U. Holm-skov, unpublished observations), are urgently needed. The demonstration of conserved binding capabilities to a broad spectrum of pathogens, as well as to endogenous proteins, by the human protein homolog also requires further investigation.

In summary, to our knowledge, the molecular and functional characteristics of a new mouse group B SRCR-SF member, SSSRCRB, have been reported in this article for the first time. Apart from its likely involvement in protection against pathogenic or saprophytic microorganismal of the restricted epithelial surfaces where mouse SSSRCRB is expressed, other functions related to epithelial cell differentiation and homeostasis should be taken into consideration for future studies.

Acknowledgments

We thank the Department of Microbiology, Hospital Clinic of Barcelona, and the Cell Biology Unit, Faculty of Medicine, University of Barcelona, for providing bacterial and fungal specimens. We also thank R. Fenutria, B. Suarez, M. Antón, C. Astasio, J. Milla, and M. Bayo for technical and administrative support.

Disclosures

The authors have no financial conflicts of interest.

References


SUPPLEMENTARY INFORMATION

Supplementary FIGURE 1. Nucleotide sequence and deduced amino acid sequence of mouse s5d-srcrb. The putative signal peptide is double underlined. SRCR domains are single underlined and the sequence with homology to syndecan domain is dash underlined. Nucleotides at the exon-intron boundaries are highlighted in bold italics. The putative N-glycosylation sites and the stop codon are marked with a circle and an asterisk, respectively.

Supplementary FIGURE 2. RT-qPCR analysis of s5d-srcrb expression in normal and leukemic cells. TRIzol total RNA preparations from the indicated mouse cells and tissues were retrotranscribed into cDNA using the SuperScript™ III First-Strand Synthesis System (Invitrogen). The primers and cycling conditions used for amplification of 18S and s5d-srcrb genes were the same as those used in Fig. 3 and reported in the Materials and Methods section. Gene expression was normalized to housekeeping gene 18S and expressed in arbitrary units. BMDMo resting, unstimulated bone marrow-derived monocytes (>95% CD11+); BMDMo LPS(+), BMDMo activated for 6 hs with 1ng/ml LPS. HEK 293 transfectants, HEK 293-EBNA cells stably expressing rmS5D-SRCRB-HA.

Supplementary FIGURE 3. Developmental expression of the s5d-srcrb transcript. Mouse embryos were obtained from CD1 mice matings and collected at 9.5 dpc. Whole-mount in situ hybridization was performed essentially as described*, using digoxigenin-labelled riboprobes. Digoxigenin was detected with NBT/BCIP (Roche) which gives a purple staining. After staining, embryos were fixed in 4% PFA,
cryoprotected in 15% sucrose and embedded in 7.5% gelatine/15% sucrose. Blocks were frozen in 2-Methylbutane (Sigma) to improve tissue preservation, then sectioned at 20 μm thickness on a Leica CM 1510-1 cryostat. Whole mount embryos and sections were imaged under a fluorescence microscope Leica DM6000B. Whole-mount in situ hybridization of 9.5 dpc embryos with s5d-srcrb antisense (a-d) and sense (e-h) probe. To generate both probes, cDNA was synthesized from total RNA amplified using primers msd1.FW and msd2.Rv cDNA was then cloned in a pFLCI vector (Qiagen). To obtain the antisense probe, the plasmid was linearized with Sall (Roche) and transcribed with T3-RNApolimerase (Roche). Similarly, for the sense probe linearization was carried out with SstI (Roche) and transcription was performed with T7-RNApolimerase. Note that specific RNA staining with antisense probe revealed s5d-srcrb expression in the anterior and lateral aspects of the otic vesicle and in the olfactory placode. nt, neural tube; ov, otic vesicle; olp, olfactory placode.


**Supplementary FIGURE 4.** RT-qPCR analysis of s5d-srcrb expression at embryo developmental stages. RNA preparations were obtained from eight different embryos of C57BL/6J mouse at 9 dpc (a, b, c and d) and 14 dpc (A, B, C and D) using TRIzol (Invitrogen). cDNA synthesis was carried out using the GeneAmp PCR kit (Roche). The primers and cycling conditions used for amplification of 18S and s5d-srcrb genes were the same as those used in Fig. 3 and reported in the Material and Methods section. Gene expression was normalized to housekeeping gene 18S and expressed in arbitrary units.
**Supplementary FIGURE 5.** Bactericidal activity was determined by a modification of the turbidimetric growth assay described by Muschel and Treffers (38). *S. aureus* at 5x10⁶ CFU/ml in TBS plus 5 mM CaCl₂ were grown alone or exposed to purified rmS5D-SRCRB-HA or BSA for 45 min in a shaker at 37°C and 280 rpm. After this time, the culture was inoculated into 5 ml LB and further incubated at 37°C and 280 rpm until turbidity measured at 600 nm in control tubes reached a DO of between 0.5 and 0.6. Serial dilutions of the cultures were then performed and plated in LB agar and the number of colonies counted in duplicate after overnight incubation at 37°C. Two independent experiments were performed, with similar results; a representative out of the two is shown here.
Supplementary Figure 2
Supplementary Figure 4
Supplementary Table I. Source of EST clones with the highest E-value for s5d-srcrb.

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dpc, days post-conception; E, embryo; EST, expressed-sequence tag;
Supplementary Table II: Reactivity of rat mAbs against recombinant and endogenous mouse S5D-SRCRB protein.

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<td>- + -</td>
</tr>
<tr>
<td>8C4.D6.C9</td>
<td>+ + +</td>
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</tr>
</tbody>
</table>

The recombinant protein comes from serum-free supernatants of HEK293-EBNA transfectants expressing a C-terminal HA-tagged mouse S5D-SRCRB protein. The tissue expressed protein comes from RIPA buffer solubilizates of mouse testis. IP, Immunoprecipitation. WB, Western blot. IHC, Immunohistochemistry.