Identification of a Novel Family of Cell-surface Proteins Expressed in Human Vascular Endothelium*

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Vascular endothelial cells (EC) play a key role in a variety of pathophysiologic processes, such as angiogenesis, inflammation, cancer metastasis, and vascular diseases. As part of a strategy to identify all genes expressed in human EC, a full-length cDNA encoding a potential secreted protein harboring 10 epidermal growth factor (EGF)-like domains and one CUB domain at the carboxyl terminus (termed, SCUBE1 for Signal peptide-CUB-EGF-like domain containing protein 1) was identified. SCUBE1 shares homology with several protein families, including members of the fibrillin and Notch families, and the anticoagulant proteins, thrombomodulin and protein C. SCUBE1 mRNA is found in several highly vascularized tissues such as liver, kidney, lung, spleen, and brain and is selectively expressed in EC by in situ hybridization. SCUBE1 is a secreted glycoprotein that can form oligomers and manifests a stable association with the cell surface. A second gene encoding a homologue (designated SCUBE2) was also identified and is expressed in EC as well as other cell types. SCUBE2 is also a cell-surface protein and can form a heteromeric complex with SCUBE1. Both SCUBE1 and SCUBE2 are rapidly down-regulated in EC after interleukin-1β and tumor necrosis factor-α treatment in vitro and after lipopolysaccharide injection in vivo. Thus, SCUBE1 and SCUBE2 define an emerging family of human secreted proteins that are expressed in vascular endothelium and may play important roles in development, inflammation, and thrombosis.

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‡ The abbreviations used are: EC, endothelial cells; EGF, epidermal growth factor; TNF-α, tumor necrosis factor-α; IL, interleukin; LPS, lipopolysaccharide; HUVEC, human umbilical vein endothelial cells; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorter; RT, reverse transcription; GADPH, glyceraldehyde-3-phosphate dehydrogenase; ECM, extracellular matrix proteins; PDGF, platelet-derived growth factor; FL, full length; TGF-β, transforming growth factor-β; LTBP, latent TGF-β-binding protein.

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Vascular endothelium (EC),1 the single layer of cells located at the interface between tissue and blood, plays an essential role in the maintenance of normal vascular physiology. Dysfunction of this cell type can lead to vascular diseases such as hypertension and atherosclerosis (1). The functional phenotype of this cell type can lead to vascular diseases such as inflammatory cytokines, growth factors, bacterial products, as well as biomechanical forces (2, 3). Many of these functions are mediated by proteins selectively expressed on the surface of ECs. For example, tissue factor expressed on the surface of EC in response to activation serves as a cofactor for factor VIIa to activate factor X and factor IX in the coagulation cascade (4). Conversely, the anticoagulant protein thrombomodulin is an EC surface molecule that binds thrombin, thereby activating protein C that in the presence of protein S degrades factor Va and VIIIa (5, 6). E-selectin is an EC-selective adhesion molecule that is rapidly induced on inflamed EC and plays a critical role in leukocyte recruitment (7). In addition, organ-selective EC surface molecules have been functionally identified in several tissues, and an endothelial marker responsible for tumor homing to the lungs has been identified (8–11). To begin to understand the repertoire of human EC surface molecules, we have combined comprehensive library sequencing with transcriptional profiling to identify EC-selective genes (12).

Approximately 100,000 cDNA fragments derived from EC were sequenced, and these sequences were then merged with public data bases to obtain ~10,000 independent gene assemblies putatively expressed in EC. To validate their endothelial expression, these genes were then represented on customized oligonucleotide microarrays (13), together with all of the non-redundant human genes from public data bases (12). Competitive hybridizations were performed utilizing both endothelial and non-endothelial cell types, and these analyses revealed ~400 genes that were uniquely expressed in EC. These included the majority of genes known to be expressed in an EC-selective pattern, such as angiotensin-converting enzyme, acetylated low density lipoprotein receptor, E-selectin, Tie-2, VEGFR2 (KDR), von Willebrand factor, NOS3, CD31, endothelin, VE-cadherin, EphB4, and ephrin-B2, and many uncharacterized genes.

One full-length cDNA identified by these approaches encoded a potential secreted protein harboring a signal peptide at the amino terminus followed by 10 EGF-like repeats and 1 CUB domain at the carboxyl terminus (termed SCUBE1 for Signal peptide-CUB-EGF-like domain containing protein 1). Interestingly, when overexpressed, SCUBE1 protein is not only secreted but is also tethered on the cell surface. Likewise, a second human gene encoding a homologue (designated SCUBE2) was also identified and appears to be expressed in EC and displayed onto cell surface in overexpressing 293T cells. SCUBE1 and SCUBE2, when singly or coexpressed, can manifest homo- and heterotypic interactions. Furthermore, SCUBE1 and SCUBE2 expression is down-regulated in EC after IL-1β and TNF-α treatment in vitro and after LPS injec-
tion in vivo, suggesting a possible role of the SCUBE gene family in the inflammatory response. Previous work has described the apparent mouse homologues of Scube1 and Scube2 (14, 15). Based solely on their expression in a variety of embryonic tissues, it was proposed that the Scube gene family may play roles in development; however, no adult expression data were reported. Our results indicate that SCUBE1 and SCUBE2 define an emerging secreted and cell-surface protein family that is expressed in human vascular endothelium.

EXPERIMENTAL PROCEDURES

In Situ Hybridization—The details of tissue preparation and in situ hybridization have been described earlier (16–18). Following the manufacturer's protocol, digoxigenin-labeled antisense and sense riboprobes were synthesized from DNA templates (nucleotides 2241–2529) using reagents supplied by Roche Molecular Biochemicals. Sectioning, pre-treatment of the sections, and hybridization of the probes were done under strict RNA-free conditions. All reagents were prepared using diethyl pyrocarbonate-treated distilled water. 15-μm thick sections were collected on positively charged slides and dried at 55 °C overnight. The sections were deparaffinized and rehydrated in Histosolve and ethanol and rinsed in diethyl pyrocarbonate-treated distilled water. The sections were treated at room temperature with 0.2 × HCl (20 min), 1.5 M NaCl (15–30 min), 0.5% Triton X-100 (15 min), followed by protease K treatment at 37 °C (30 min). The sections were then washed with triethanolamine buffer followed by acetylation with acetic anhydride. Following prehybridization in 2× SSC, containing 50% formamide at 37 °C (1 h), the sections were air-dried at room temperature. Sections were hybridized with the probes diluted in hybridization solution (2× SSC) and finally in 0.1× SSC at room temperature, 0.2°C for 15 min. The sections were washed with triethanolamine, pH 7.4, 0.005% sodium pyrophosphate, and 500 μg/ml yeast tRNA at 55 °C overnight. Following hybridization, the sections were washed with 4× SSC (twice for 15 min) and 2× SSC (twice for 15 min). The sections were then treated with RNase A at 37 °C (30 min) followed by stringency washes in 2× SSC at 37 °C (15 min), 0.1× SSC at 42 °C (40 min) and finally in 0.1× SSC at room temperature, 0.2°C for 15 min. The sections were washed with maleate buffer (30 min) and then blocked with 10 mM Tris buffer, pH 7.6, containing 500 μM NaCl, 4% bovine serum albumin, 0.5% cold-water fish skin gelatin, and 0.05% Tween 20. The sections were then incubated with anti-digoxigenin antibody, conjugated to peroxidase (Roche Molecular Biochemicals), for 1 h. The signal was amplified using TSA-Plus kit (PerkinElmer Life Sciences), and the signal was detected with Vector Blue substrate (Vector Laboratories, Burlingame, CA). Following incubation with substrates the sections were dehydrated in ethanol and Histosolve and coverslipped. Hybridization with the sense control probe did not result in detectable signal, indicating the specificity of hybridization. All sections were observed with a Nikon BX50 microscope (Olympus, Inc., Melville, NY), using DIC illumination. The microscope was equipped with a Nikon DXM1200 digital camera (Technical Instruments, San Francisco, Burlingame, CA). Digitized images (1280 × 1024-pixel resolution) were acquired using ACT-1 software (Nikon, Melville, NY). Images were resized, cropped, and assembled using Photoshop version 6.0 (Adobe Systems, San Jose, CA). Apart from equalizing the background intensities, no other digital modifications of the original digital images were carried out.

Identification of the Full-length Clones of Human SCUBE1 and SCUBE2—To capture the diverse repertoire of genes expressed in EC, multiple libraries from EC under various stimuli (such as shear-stress, proinflammatory cytokine-treated) were generated using methods described previously (19). Approximately 100,000 cDNA fragments were sequenced, and these sequences were normalized using methods described previously (19). Approximately 100,000 cDNA fragments were sequenced, and these sequences were then merged with public data bases to obtain 10,000 EC gene independent assemblies. To validate their endothelial expression, these genes were represented on custom microarray (5). The GCA TGG GCG CGG-3′ and 5′-CCC GGT TAT TAT TGG GCG GCAG AAC C3′ were used to amplify the entire open reading frame by PCR from a mixture of human cDNA libraries. The amplified SCUBE1 full-length cDNA was cloned into pcR2.1 (Invitrogen) and confirmed by sequencing. The clone containing full-length SCUBE2 was obtained from Origene Technologies (Rockville, MD). Full-length sequences for SCUBE1 were deposited into GenBank™ with accession number AF525689, and the SCUBE2 sequence is the same as NM_020974 (except nucleotides 1287 to 1526 are spliced out in the clone we used).

Northern Blot Analysis—The human Northern blot was purchased from Clontech and hybridized with a radiolabeled human SCUBE1 cDNA probe (nucleotides 2077–2851) per the manufacturer’s protocol. Construction of Expression Plasmids—The epitope-tagged versions of SCUBE1 or SCUBE2 were constructed in the following expression vectors. The pCDNA4/Myc-His (Invitrogen) was used to add a Myc tag to the carboxyl terminus of SCUBE1 containing endogenous signal peptide. Alternatively, premade first-strand cDNAs from human tissues (Clontech) were used to add a Myc tag at the carboxyl terminus of SCUBE2. The pFLAG-CMV-1 (Sigma) was used to include a FLAG tag at the amino terminus of target protein.

Cell Culture and Transfection—Human embryonic kidney 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin. Cells were seeded in 6-well plates overnight before transfection. The transfection was performed by using FuGENE 6 reagent (Roche Molecular Biochemicals). The total amount of DNA was kept constant in all transfections by supplementing empty vector DNA.

Human umbilical vein endothelial cells (HUVEC) were cultured as described (14). A total concentration of 1 mg/ml. LPS solution (5 mg/kg) or PBS was injected intraperitoneally into a group of three C67BL/6 mice, and at the indicated times, animals were harvested, washed in sterile cold PBS, and frozen in liquid nitrogen.

RNA Isolation, Semi-quantitative Reverse Transcription (RT)-PCR, and TagMan Analyses—Total RNA was prepared from cultured cells or harvested animal kidneys using TRIzol reagents (Invitrogen). First-strand cDNA synthesis using SuperScript II reverse transcriptase (Invitrogen) was performed on 5 μg of total RNA. One-tenth of the first-strand cDNA reaction was used for PCR as template. Alternatively, premade first-strand cDNAs from human tissues (Clontech, Palo Alto, CA) were used as PCR template. Semi-quantitative RT-PCR primers specific for human SCUBE1 and SCUBE2 were as follows: SCUBE1-f2, AGT GGT CTC CAG CAC TCT TCT; SCUBE1-r2, confirmed by sequencing. The clone containing full-length SCUBE2 was obtained from Origene Technologies (Rockville, MD). Full-length sequences for SCUBE1 were deposited into GenBank™ with accession number AF525689, and the SCUBE2 sequence is the same as NM_020974 (except nucleotides 1287 to 1526 are spliced out in the clone we used).

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CAG TGC TGG TTT TTG CAG TGT; SCUBE2-f2, AGA CCC CAG AAG CTT GGA ATA; SCUBE2-r2, TCC CCT CCA CAT CTT CTG TTT. GAPDH primers were obtained from Clontech. Real-time TaqMan PCR analyses were performed using Applied Biosystems PRISM 7700 Sequence Detection System. Normalization was performed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels as controls in parallel TaqMan reactions. The 5′/H11032 and 3′/H11032 primers and a fluorescence-labeled probe were designed as follows: SCUBE1 5′/H11032 primer, AAC ACA CGG GTA CCG CCT CTT; SCUBE1 3′/H11032 primer, GTA TTG TAG TGG TGT CCG GGA GA; SCUBE1 probe, CCA GGA CTG CGA GGC CAA AGT GCA T; SCUBE2 5′/H11032 primer, CAG GAT TGT GAA ACC CGA GTT C; SCUBE2 3′/H11032 primer, CGG ATA CAT CGG TGT GTG GTG; SCUBE2 probe, TGC TCG CCT GGG CAT TTC TAC AAC A; GAPDH primer-probe was purchased from BioSource International (Camarillo, CA).

RESULTS

Identification of a Gene Highly Expressed in EC—The endothelial origin of a SCUBE1 cDNA fragment identified by genomic approaches was confirmed by in situ hybridization (Fig. 1). It was expressed in the luminal endothelial cells of the human umbilical vessels (Fig. 1, inset). Furthermore, localization of this gene to the EC was demonstrated in human umbilical cord artery (a), lung (b), and kidney (c and d), and monkey brain (b), lung (c and d), and kidney (c and d). In addition to endothelial expression in artery and vein, this gene is also expressed in microvascular endothelial cells in a variety of tissues (Fig. 1, e and f, and data not shown).

To obtain the full-length cDNA of this gene, the original cDNA fragment was mapped to human genomic sequence (www.ensembl.org) and was found to be localized on chromosome 22q13, where a human gene was predicted based on its homology to mouse Scube1 (14). Two oligonucleotides, based on this gene prediction, were used to amplify the entire open reading frame from human cDNAs. This cDNA contains an open reading frame of 2964 nucleotides and encodes a polypeptide of 988 amino acids (Fig. 2a). Hydropathy (21) and protein family analyses (22) predict one amino-terminal signal peptide (22 amino acids) followed by 10 EGF-like repeats and a CUB domain (Fig. 2). There is an apparent spacer region located between the 9th and 10th EGF-like repeats (Fig. 2b). This domain structure is identical to that of mouse Scube1, therefore, this human orthologue was designated SCUBE1 to be consistent with the literature (14). Mature SCUBE1 is predicted to contain 966 amino acids with calculated molecular mass of 106 kDa.

Tissue Distribution of Human SCUBE1 Transcript—A Northern blot containing poly(A)+-enriched mRNA (2 μg) from a variety of human adult tissues was hybridized with a human SCUBE1 cDNA radiolabeled probe. The expression level of the SCUBE1 transcripts was highest in liver, kidney, lung, and small intestine, followed by brain, colon, and spleen. The expression in remaining tissues was very low or undetectable (Fig. 3). Expression of SCUBE1 in several highly vascular tissues, such as liver, kidney, and lung, is consistent with the endothelial origin of SCUBE1 demonstrated by in situ hybridization (Fig. 1). The size of the primary transcript for SCUBE1 (4 kb) is consistent with both the predicted and cloned full-length cDNA (Fig. 2).

SCUBE1 Is a Secreted and Cell-associated Protein—Because
human SCUBE1 protein has a putative signal peptide at the amino terminus and because human SCUBE1 protein contains 10 EGF-like repeats that are found in many extracellular matrix proteins (ECM) (23, 24), we examined whether the SCUBE1 protein is a secretory and/or ECM protein. For this purpose, recombinant SCUBE1 protein was expressed by means of transient expression in human embryonic kidney 293T cells. The Myc epitope tag was added at the carboxyl terminus for the detection of the recombinant protein. Two days after transfection, the culture supernatants were collected, and cells were detached from dishes by EDTA treatment, and residual ECM proteins were extracted with Laemmli buffer. Samples collected from these three fractions were subjected to Western blot analyses using anti-Myc antibody. As shown in Fig. 4a, human SCUBE1 protein was detected in the conditioned cell culture medium (Medium) and in cells (Cell) but was not detected in the ECM fraction (Matrix) or in fractions from the control vector-transfected cells. These data demonstrate that the SCUBE1 protein is a secreted and cell-associated protein.

Because the CUB domain was recently described in two novel members of the platelet-derived growth factor (PDGF) family that require proteolytic activation (25–28), we tested whether secreted SCUBE1 could be subject to proteolytic cleavage. A dual epitope-tagged SCUBE1 with FLAG and Myc tags added to the amino and carboxyl terminus, respectively, was transiently expressed in 293T cells. Forty eight hours post-transfection, samples from conditioned medium, cells, and ECM were individually immunoblotted with anti-FLAG or anti-Myc antibodies. As shown in Fig. 4b, the molecular weight of detected SCUBE1 protein is identical either by anti-FLAG or anti-Myc antibodies. These results suggest that the secreted SCUBE1 protein does not undergo further proteolytic processing. Consistent with this finding, the dual-tagged SCUBE1 protein immunoprecipitated by FLAG antibody from cell culture conditioned medium (Medium) showed the identical molecular size when blotted either with anti-FLAG or Myc antibodies separately (Fig. 4c).

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glycosylation motifs (Fig. 2a), we hypothesized that SCUBE1 is subject to post-translational modification by glycosylation. Thus, we examined whether tunicamycin, an inhibitor of N-glycosylation, affected the molecular size of the protein. As shown in Fig. 5, tunicamycin treatment of cells resulted in a reduction in the molecular size of the precursor form of the SCUBE1 protein (lanes 2 and 5), indicating that the majority of SCUBE1 is glycosylated when expressed in 293T cells. To determine the contribution of the six putative N-linked glycosylation sites in the SCUBE1 protein, we compared the molecular size of the carboxyl-terminal deletion mutants (D1 and D2) with or without tunicamycin treatment. As shown in Fig. 5, the precursor form of mutant D1 (in which the carboxyl-terminal CUB domain is deleted) was detected at 110 kDa without tunicamycin (lane 3), whereas the protein size was shifted to a faster migrating band by treatment with tunicamycin (lane 6), indicating that the mutant D1 is glycosylated. However, tunicamycin treatment did not change the apparent molecular size of the precursor form of mutant D2 (lanes 4 and 7) (in which five of six putative N-linked glycosylation motifs in the protein are deleted) (Fig. 2b). Taken together, these data demonstrated that SCUBE1 is N-glycosylated at multiple sites within the carboxyl-terminal region.

Homomeric Interactions of SCUBE1 Proteins—Because families of secreted growth factors or cytokines are often capable of forming dimeric or higher ordered complexes (29, 30), and because SCUBE1 is a secretory protein, we hypothesized that oligomeric forms of SCUBE1 protein may exist. We constructed cDNAs encoding FLAG- or Myc-tagged SCUBE1 proteins, and we examined their association by co-immunoprecipitation assays from both singly and co-transfected 293T cells (Fig. 6a). Lysates of these cells were immunoprecipitated with the anti-Myc monoclonal antibody, and then the precipitates were analyzed by immunoblotting with anti-FLAG monoclonal antibody. A 130-kDa immunoreactive band recognized by anti-FLAG antibody was observed in the anti-Myc immunoprecipitates from cells co-expressing SCUBE1-Myc and Flag SCUBE1 proteins but not from cells transfected with individual tagged constructs alone (Fig. 6a). Likewise, the reciprocal immunoprecipitation of Flag SCUBE1 results in the co-precipitation of SCUBE1-Myc (Fig. 6a). We did not observe an association between SCUBE1 and IL-1R1, suggesting specificity of the homomeric interactions between SCUBE1 proteins (Fig. 6a). Furthermore, homomeric association of SCUBE1 proteins appears to require co-expression and is not an artifact formed only after cell lysis, because a mixture of lysates containing separately expressed tagged proteins is not sufficient for complex formation in this heterologous expression system (data not shown). These results demonstrate that human SCUBE1 proteins are capable of forming oligomeric complexes when co-expressed.

We then used carboxyl-deletion mutants (D1 and D2) to characterize which domains of SCUBE1 are necessary for SCUBE1 oligomerization. Two deletion constructs, FLAG-tagged SCUBE1-D1 or SCUBE1-D2 and full-length (FL) SCUBE1, were transiently co-expressed with SCUBE1-Myc in 293T cells (Fig. 6b). Immunoprecipitation of SCUBE1-Myc resulted in the co-precipitation of Flag SCUBE1-D1 or Flag SCUBE1-D2 (Fig. 6b). These data suggest that the first nine copies of the EGF-like repeats in the SCUBE1-D2 mutant protein are sufficient for SCUBE1 complex formation.

**Human SCUBE1 Is a Peripheral Membrane Protein**—Be-
cause the majority of expressed SCUBE1 protein appears cell-associated (Fig. 4a), we next determined its subcellular distribution by biochemical fractionation. 293T cells transiently expressing FLAG-tagged SCUBE1 proteins were lysed in hypotonic buffer, and low and high speed centrifugation was performed to obtain a membrane fraction (P100) and a cytoplasmic fraction (S100). Subcellular distribution of SCUBE1 protein was monitored by anti-FLAG immunoblotting. As shown in Fig. 7, most of the Flag.SCUBE1 protein was partitioned into the P100 membrane fraction, suggesting that SCUBE1 is membrane-bound. The presence of a small amount (less than 10%) of SCUBE1-D2 mutant protein in the cytosol may be due to some aberrant processing of this mutant or simply be a nonspecific effect of overexpression.

To assess the physical nature of SCUBE1 interaction with the plasma membrane, the P100 membrane fraction was treated with conditions that solubilize peripheral membrane proteins. All three forms of SCUBE1 protein were stripped off the membrane when incubated with 0.1M Na2CO3, pH 12 (Fig. 7, S100/H11032), whereas the Toll-like receptor-2 (TLR2), a single membrane-spanning receptor (31), was not released from the washed membranes (Fig. 7, P100/H11032). These data indicate that membrane-bound SCUBE1 behaves like a peripheral membrane protein.

Spacer Region Is Critical for SCUBE1 Protein Secretion and Cell-surface Expression—Because expression of FL SCUBE1 can result in a secreted protein, we examined whether or not the carboxyl domain plays a role in protein secretion. Two carboxyl-terminal deletion constructs, SCUBE1-D1 or SCUBE1-D2, were transiently expressed in 293T cells. The conditioned culture medium was collected and subjected to Western blotting analysis using anti-FLAG antibody. As shown in Fig. 8a, SCUBE1-D1 mutant (deleting the CUB domain) was expressed and secreted into the conditioned medium like the FL protein, whereas the SCUBE1-D2 mutant protein was not secreted (Fig. 8a). These data suggest that the spacer region, located between the 9th and the 10th EGF-like motif, is essential for SCUBE1 protein secretion, at least when overexpressed in 293T cells.

To confirm that SCUBE1 is a secreted, peripheral membrane protein, we examined SCUBE1 cell-surface expression by flow cytometry analysis in intact cells. As shown in Fig. 8b, we confirmed the cell-surface expression of wild-type SCUBE1-FL or SCUBE1-D1 proteins (top and middle) by FACS analysis,
whereas the SCUBE1-D2 deletion mutant is defective in cell-surface targeting when individually expressed (bottom). However, because SCUBE1-D2 mutant protein is capable of forming complexes with wild-type protein (Fig. 6b) and because wild-type SCUBE1-FL is a cell-surface protein (Fig. 8a), we next tested if co-expression with FL protein can restore the cell-surface targeting phenotype of SCUBE1-D2 mutant. Indeed, as shown in Fig. 8b (bottom), co-expression of wild-type SCUBE1.Myc resulted in the expression and targeting of SCUBE1-D2 mutant onto the cell surface as determined by flow cytometry.

Identification of a Second Member of SCUBE Family—By utilizing homology searches, we identified a homologous human gene encoding a protein with identical domain structure to that of SCUBE1, designated SCUBE2. The mouse orthologue (Scube2) of this human gene was recently described (15). To compare the tissue expression patterns of human SCUBE1 and SCUBE2, each gene was examined by semi-quantitative PCR in a panel of human tissue cDNA (Fig. 9). Consistent with the Northern blot analysis (Fig. 3), SCUBE1 mRNA expression was restricted to few highly vascularized tissues such as liver, lung, and kidney (Fig. 9). In contrast, SCUBE2 transcript was expressed in a broad spectrum of human tissues (Fig. 9). Likewise, SCUBE2 message, but not SCUBE1, was also observed in several non-endothelial human primary cell types such as fibroblasts and renal mesangial cells (Fig. 10).

The co-expression of the closely-related SCUBE1 and SCUBE2 mRNAs in cultured ECs (Fig. 10) raises the question of whether these proteins interact. To ascertain the formation of heteromeric complexes, differentially epitope-tagged SCUBE1 and SCUBE2 constructs were singly or co-transfected into 293T cells, and immunoprecipitations were performed. When Flag.SCUBE2 is co-expressed with either SCUBE1.Myc or SCUBE2.Myc, immunoprecipitation with FLAG antibody co-precipitates both proteins (Fig. 11). This interaction is not seen with tagged IL-1R1, indicating the specificity of this assay (Fig. 11).

Down-regulation of the SCUBE Transcripts by Proinflammatory Cytokines in Vitro and LPS in Vivo—Because vascular ECs are important cellular targets for the actions of proinflammatory cytokines (32), we determined whether or not expression of the endothelial Scube gene family could be altered in response to proinflammatory cytokines. Cultured HUVEC were exposed to IL-1β/H9252 or TNF-α/H9251 for 6 and 24 h, respectively. Total RNAs isolated from cytokine-treated HUVEC were subjected to semi-quantitative RT-PCR to measure the expression level of SCUBE1 and SCUBE2. As shown in Fig. 10, SCUBE1 transcript was significantly down-regulated by both cytokine treatments with IL-1β stimulation, demonstrating a more rapid response than that of TNF-α treatment. In contrast, SCUBE2 expression was only modestly depressed in response to 24 h of TNF-α treatment.

To validate further this observation in vivo, we investigated whether the expression of SCUBE1 and SCUBE2 was affected by systemic LPS administration in C57BL/6 mice. A group of animals (n = 3) was sacrificed after intraperitoneal injection of...
either LPS (5 mg/kg) or PBS vehicle at 3, 6, 24, and 48 h.
Kidneys were collected and subjected to real time TaqMan analyses. As shown in Fig. 12, SCUBE1 RNA levels were dramatically down-regulated 3 h after injection of LPS and then quickly returned to untreated levels. SCUBE2 expression was also depressed by LPS injection, although with slightly slower kinetics, reaching the lowest level at 6 h postinjection and then recovering to untreated control levels over a 48-h period. These results indicate that both the \textit{Scube1} and \textit{Scube2} genes are dynamically responsive to inflammatory stimuli in vivo.

\section*{DISCUSSION}

In this report we have identified a family of secreted proteins expressed in human vascular endothelial cells. Members of this gene family were named as SCUBE, based on their unique domain structures which consist of one Signal peptide, one CUB domain, and multiple EGF-like repeats (Fig. 2). SCUBE1 appears to be expressed selectively in endothelium. \textit{In situ} hybridization demonstrated SCUBE1 expression in the EC lining the arteries, veins, and the microvessels of all organs examined (Fig. 1). Consistent with this finding, the highest levels of SCUBE1 expression are found in vascularized tissues (Fig. 3). In contrast, SCUBE2 is expressed in EC but is also found in additional cell types such as fibroblasts and renal mesangial cells and is expressed in a wide range of tissues (Figs. 9 and 10). Based on these disparate expression patterns, the SCUBE proteins may mediate distinct sets of functions in these various cell types and tissues.

The EGF-like repeat, a six-cysteine conserved motif, is found in many classes of proteins such as secreted growth factors, transmembrane receptors, adhesion molecules, signaling proteins, and components of the ECM (24). On the other hand, the CUB domain, which was named based on the first three identified proteins of this family, \textit{i.e.} complement proteins C1r/C1s, Uegf (sea urchin fibropellins), and Bmp1, is thought to mediate protein-protein interaction and has been found in a more limited set of proteins involved in developmental processes such as embryogenesis or organogenesis (33). A number of proteins with a role in embryonic development or immune responses have been identified that contain both the EGF-like and the CUB domains. These include \textit{Drosophila} tolloid (34) and the mammalian tolloid-like proteins \textit{BMP1} (procollagen C proteinase) (35) and \textit{mTll} (36), sea urchin fibropellins (37), the complement proteins C1s and C1r (37, 38), and the serum glycoprotein attractin (39, 40). Mouse \textit{Scube1} had been reported to be expressed prominently in the developing gonad, nervous system, somites, surface ectoderm, and limb buds (14), whereas mouse \textit{Scube2} was restricted to the embryonic neuroectoderm in mouse embryos ranging from 7.5 to 12.5 days post-coitum (15). Based on these expression patterns, it was hypothesized that the \textit{Scube} gene family may play roles in the development of these organ systems (14, 15). In our studies, we have observed pan-endothelial expression of \textit{Scube1} in mouse 17.5 days post-coitum embryos by \textit{in situ} hybridization (data not shown).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig10.png}
\caption{Down-regulation of the SCUBE1 and SCUBE2 messages upon the treatment of proinflammatory cytokines in HUVEC. Several human primary cells and HUVEC treated with IL-1\beta and TNF-\alpha for 6 and 24 h were submitted for RT-PCR analyses. AOSMC, aortic smooth muscle cell.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig11.png}
\caption{Hetero- and homo-oligomerizations of SCUBE2 in transfected 293T cells. Indicated expression plasmids were singly or co-transfected in 293T cells. Detergent lysates of each transfection were subjected to immunoprecipitation (IP) and immunoblotting (WB) using antibodies as indicated. Experiments were performed twice with similar results. The double bands seen for the Flag,SCUBE1 and Flag, IL-1R1 are due to the glycosylation of these proteins.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig12.png}
\caption{Depression of the \textit{Scube} gene family expression upon the treatment of LPS in mice. A group of C57/BL6 mice (\textit{n} = 3) were sacrificed after intraperitoneal injection of either LPS at 5 mg/kg or PBS vehicle at indicated time points. Kidneys were collected and submitted for the TaqMan analyses as described under “Experimental Procedures.”}
\end{figure}
shown). These results are consistent with the pan-endothelial expression of SCUBE1 in the adult (Fig. 1). Thus, it appears that the endothelial restricted expression of this gene is acquired relatively late in development and persists into adulthood. These data suggest that this protein may have critical roles in both development and endothelial/vascular biology in the adult.

Based on cellular and biochemical studies, we have demonstrated that SCUBE1 proteins are capable of forming homomeric complexes and that the first nine EGF-like repeats are sufficient to maintain these interactions in 293T cells (Fig. 6). Because of the presence of both SCUBE1 and SCUBE2 in EC, we further investigated whether heteromeric complexes could form. These studies have indicated that both SCUBE1 and SCUBE2 can form homo- and heteromeric complexes with each other (Figs. 6 and 11). These data suggest that SCUBE1 and SCUBE2 may have related functions in EC.

The molecular mechanisms by which SCUBE proteins target to the cell surface and associate with the membrane remain unclear at present. We originally hypothesized that a short hydrophobic stretch within the CUB domain (Fig. 2b) may be responsible for the cell-surface association. However, this was disproved because the SCUBE1-D1 deletion mutant, which lacks the carboxyl CUB domain, still retains its ability to be secreted and cell surface-associated (Figs. 7 and 8). Surprisingly, the spacer region, located between the 9th and 10th EGF-like repeats, appears essential for SCUBE1 protein secretion and cell-surface targeting, because the SCUBE1-D2 deletion mutant, which is missing this spacer region as well as the rest of the carboxyl portion, is defective in secretion and cell-surface expression (Fig. 8). Interestingly, the defective phenotype of SCUBE1-D2 mutant protein can be effectively rescued by co-expression with FL SCUBE1 (Fig. 5b). This is consistent with the finding that SCUBE1-D2 mutant protein is capable of forming a complex with the wild-type SCUBE1-FL protein (Fig. 5b) and suggests that this physical interaction with the wild-type protein may be playing a role in the “re-targeting” to cell surface (Fig. 5b). SCUBE1 is a glycoprotein (Fig. 5), and the deletion of multiple N-linked glycosylation sites in SCUBE1-D2 deletion mutant resulted in a loss of cell surface association (Fig. 8b). Thus, it may be that yet-to-be-identified lectins or mucin-like proteins with carbohydrate-binding capacity could serve as receptor(s) or binding site(s) for SCUBE proteins on EC. Alternatively, the EGF-like repeats of SCUBE proteins form heteromeric complexes with other cell-surface proteins containing EGF-like repeats, much like the interactions seen in the transmembrane Notch receptors and their ligands, Delta and Serrate (41).

Data base searches identified members of the fibrillin family of ECM proteins as having significant homology to SCUBE proteins, based largely on their multiple EGF-like repeats. Interestingly, mutations in the fibrillin-1 gene cause Marfan syndrome, which is characterized in part by significant vascular abnormalities (42). Another protein family that contains multiple EGF-like repeats is the latent TGF-β-binding protein (LTBP) family. Four different LTBP homologues, LTBPs 1–4, have been characterized (43, 44). LTBPs function to enhance secretion and stability of the latent TGF-β complex, ensure correct folding of TGF-β, and target the latent TGF-β complex to the ECM of certain cells and tissues for storage or to the cell surface where activation takes place (43, 45, 46). To examine if SCUBE1 could have a similar role, we tested whether SCUBE proteins, like LTBPs, are capable of binding growth factors or cytokines. Flag-SCUBE1 was co-expressed individually with PDGF-D, IL-8, or IL-17F, all of which are expressed in or have functions in EC (27, 28, 47, 48). In our primary studies, Flag-SCUBE1 proteins were capable of forming stable complexes with PDGF-D and IL-17F but not with IL-8 (data not shown). Although the full significance of this interaction is currently unknown, this observation suggests that SCUBE1 proteins may potentially function to modulate the expression or function of certain growth factors.

An additional interesting domain feature of SCUBE1 is that it contains six Ca$^{2+}$-binding EGF-like repeats (49), a motif found in many coagulation factors (e.g. VII, IX, and XII) (50, 51) as well as anticoagulant proteins (e.g. thrombomodulin and protein C) (52, 53). As shown in Figs. 10 and 12, SCUBE1 and SCUBE2 transcripts were down-regulated in EC either by inflammatory cytokines in vitro or LPS injection in vivo. This is reminiscent of the suppression of EC cell-surface thrombomodulin and protein C expression, in response to IL-1β, TNF-α, and LPS treatment (54–58). These data strongly suggest that SCUBE proteins are involved in the inflammatory responses and raise the interesting hypothesis that they may be modulators of thrombosis or coagulation.

In summary, we have identified a novel family of human EC-expressed secreted proteins termed SCUBE proteins. SCUBE1 appears to represent a novel Pan-endothelial expressed protein, and both SCUBE1 and SCUBE2 are found in EC. Although the precise functions are currently unknown, their unique structures, combined with their patterns of expression and modulation by inflammatory stimuli, point to potential roles in development, inflammation, and thrombosis.

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Novel Family of Endothelial Cell-surface Proteins 46373
Identification of a Novel Family of Cell-surface Proteins Expressed in Human Vascular Endothelium

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