We have previously identified a novel family of secreted, cell-surface proteins expressed in human vascular endothelium. To date, two family members have been described, sharing a characteristic domain structure including an amino-terminal signal peptide followed by multiple copies of epidermal growth factor (EGF)-like repeats, a spacer region, and one CUB domain at the carboxyl terminus. Thus, this family was termed SCUBE for signal peptide-CUB-EGF-like domain containing proteins. Here we described the identification and characterization of one additional member of the SCUBE family named SCUBE3 in humans, sharing an overall 60% protein identity and a similar domain structure with other family members. Real-time quantitative reverse transcriptase-PCR and Northern blot analyses revealed that this gene is highly expressed in primary osteoblasts and the long bones (humerus and femur), followed by a low level of expression in human umbilical vein endothelial cells and in heart. When overexpressed in human embryonic kidney 293T cells, the recombinant hSCUBE3 protein is a secreted glycoprotein that can form oligomers tethered to the cell surface. Interestingly, the secreted hSCUBE3 protein can be further proteolytically processed by a serum-associated protease to release the EGF-like repeats from the CUB domain. The SCUBE3 gene is mapped to human chromosome 6p21.3, a region that has been linked with the locus for a rare form of metabolic bone disease, Paget’s disease of bone 1. Together, this novel secreted, cell-surface osteoblast protein may act locally and/or distantly through a proteolytic mechanism, and may play an important role in bone cell biology.

We have previously utilized a combination of high-throughput sequencing and genome-scale microarray expression profiling to identify novel cell-surface proteins expressed in human umbilical vein endothelial cells (1, 2). One gene family identified by this approach encodes potential secreted proteins harboring a signal peptide at the amino terminus followed by multiple copies of EGF-like repeats and one CUB domain at the carboxyl terminus (2). Thus, these genes were termed SCUBE for signal peptide-CUB-EGF-like domain containing proteins. Expression of the first gene member SCUBE1 is highly enriched in endothelial cells, whereas the second gene member SCUBE2 is expressed in endothelial cells as well as other cell types (2). The recombinant SCUBE protein, when overexpressed in human embryonic kidney 293T (HEK-293T) cells, is a secreted glycoprotein that can form oligomers and manifests a stable association with the cell surface (2). Both SCUBE1 and SCUBE2 are rapidly down-regulated in endothelial cells after interleukin-1β and tumor necrosis factor-α treatment in vitro and after lipopolysaccharide injection in vivo, suggesting a possible role for the SCUBE gene family in the inflammatory response (2). In the present study, we identified and characterized the third member of the SCUBE gene family, named SCUBE3. Its unique expression in bones and osteoblasts suggests that SCUBE3 may play a critical role in bone cell biology.

EXPERIMENTAL PROCEDURES

Reagents—Human primary cultured cells, osteoblasts, human umbilical vein endothelial cells (HUVEC), and coronary smooth muscle cells, were purchased from Cambrex Bio Science (Walkersville, MD). TaqMan Universal PCR master mixture was from PE Applied Biosystems (Foster City, CA).

Long Bone RNA Extraction and Analysis—The long bones (humeri and femurs) from one 2-month-old male C57BL6 mouse were dissected free of surrounding tissue, cut into small pieces in 2 ml of TRIzol reagent (Invitrogen) and homogenized with a Polytron probe (Brinkmann Instruments) for 30 s. Total RNAs were prepared using TRIzol reagent according to the manufacturer’s instructions. 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 as template for each real-time quantitative RT-PCR analysis as described below.

Real-time Quantitative RT-PCR (TaqMan) Analysis—hSCUBE3 mRNA expression was measured by real-time quantitative RT-PCR on a panel of cDNAs from a variety of human primary cells or normal tissues. Probes were designed by PrimerExpress software (PE Applied Biosystems) based on the sequence of the hSCUBE3 gene. The hSCUBE3 gene-specific probe was labeled using FAM (6-carboxyfluorescein) and the β2-microglobulin reference probe was labeled with a different fluorescent dye (VIC) at the 5′ end. TAMRA (6-carboxy-N,N,N′,N′-tetramethylrhodamine) is linked at the 3′ end as quenchers. The differential labeling of the target gene and internal reference gene

The abbreviations used are: EGF, epidermal growth factor; RT, reverse transcriptase; TAMRA, 6-carboxy-N,N,N′,N′-tetramethylrhodamine; h, human; m, murine; HUVEC, human umbilical vein endothelial cells; FBS, fetal bovine serum; ECM, extracellular matrix; PDB, Paget’s disease of bone.

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Bo-Tsung Wu‡, Yueh-Hsing Su‡, Ming-Tzu Tsai‡, Scott M. Wasserman‡, James N. Topper‡, and Ruey-Bing Yang‡

From the ‡Institute of Biomedical Sciences, Academia Sinica, Taipei 11529, Taiwan, and †Division of Cardiovascular Medicine, Stanford University School of Medicine, Stanford, California 94305, and ‡Frazier Healthcare Ventures, Palo Alto, California 94301
thus enabled measurement in the same well. Normalization was per-
formed using β2-microglobulin mRNA levels as controls in the same
reaction. TaqMan experiments were carried out in an ABI PRISM 7700
Sequence Detection System (PE Applied Biosystems) using TaqMan
reagents. The thermal cycler conditions were as follows: hold for 2 min
at 50 °C and 10 min at 95 °C, followed by two-step PCR for 40 cycles of
95 °C for 15 s followed by 60 °C for 1 min. Primers used for human (h)
SCUBE3 analysis were as follows: forward, 5′-CAAAGTCCTGGCTC-
CCAG and reverse, 5′-GACGGACTGGCGGTCTGATAG; TaqMan probe,
5′-FAM-ACACCATCACCACCTGGTATCG-TAMRA. Primers
used for mouse (m) SCUBE3 analysis were as follows: forward, 5′-CCA-
GAAGAAGGAGTGAAGCTCA and reverse, 5′-CAATGGCCG-
CCTTGG; TaqMan probe, 5′-FAM-TGGCCCAATTCTGGCAGGACT-
TAGC. Primers used for β-actin microglobulin analysis were as follows:
forward, 5′-GTCTGCTCCCTGGTGCCCTTA and reverse, 5′-TGATCTT-
TGAGATCCGCTGGA; TaqMan probe, 5′-VIC-TGCTCCGCTAC-
CTTTCCTTCTGCG-TAMRA. Primers used for GAPDH analysis were as
follows: forward, 5′-TGAGGCTGAGTCAACCG and reverse, 5′-A-
GAGTTAAAAGCAGCCCTGTTGG; TaqMan probe, 5′-FAM-TTGTG-
CTATTGGCGCCTGG-TAMRA.

**Full-length Cloning and Expression Plasmids**—On the basis of gene
prediction and a public sequence information (GenBank accession
number AF452494), the entire open reading frame of hSCUBE3 was
amplified by PCR via a mixture of Advantage 2 (Clontech) and
Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA) from cDNAs derived
from a panel of human primary osteoblasts. The resulting cDNA was cloned into
pGEM-T Easy vector (Promega) and confirmed by sequencing. The
following epitope-tagged expression constructs of hSCUBE3 were pre-
pared by cloning of the PCR fragment encoding the mature hSCUBE3
(amino acids 23–985) into the mammalian expression vectors. The
FLAG tag (DYKDDDDK) was added at the amino terminus followed by
to add a Myc tag (EQKLISEEDL) at the carboxyl terminus of
hSCUBE3. The GenBank accession numbers for hSCUBE3 and
mSCUBE3 are AF452494 and AF452495, respectively.

**Northern Blot Analysis**—Total RNAs (10 μg) from human oste-
oblasts and human small intervertebral discs were separated on 1.2%
denaturing formaldehyde-agarose gel, transferred to nylon membranes, and
hybridized overnight with a radioiodinated 525-bp hSCUBE3 cDNA
probe (amino acids 356–531). In addition, one human tissue Northern
blot (containing 1 μg of poly(A)-enriched mRNA from a variety of human tissues) was purchased from Clontech and hybridized with a
radioiodinated hSCUBE3 cDNA probe according to the manufacturer’s
instructions. After hybridization overnight, the blots were washed once
at room temperature (25 °C) and twice at 65 °C with 0.1 × SSC (15 mM
NaCl, 1.5 mM sodium citrate) and 0.1% SDS (each wash for 1 h).

**RESULTS**

**Identification and Expression Profile of Human SCUBE3**—
We have previously identified a family of cell-surface proteins
expressed in human endothelial cells (2). To date, two gene
members have been described sharing a characteristic domain
structure including an amino-terminal signal peptide, followed by
multiple copies of EGF-like repeats, a spacer region, and one
CUB domain at the carboxyl terminus (2). Thus, this family
was termed SCUBE.

To further understand the biology of this gene family, we
investigated whether or not additional SCUBE family mem-
ber(s) exist in the public sequence data bases. After searching a
panel of non-skeletal cDNA data bases (GenBank accession XM
094924), homologous to SCUBE1 and SCUBE2, was identified on human chromosome 6p21. Subsequently, two
independent cDNAs representing this predicted gene were
described in the GenBank data base under accession numbers
AF452494 and BC052263, respectively. In this report, this gene is
referred to as SCUBE3 to be consistent with the literature and
the order of its discovery, related to prior family members,
SCUBE1 and SCUBE2 (2, 4, 5).

We then examined the expression profile of human SCUBE3
(hSCUBE3) by real-time quantitative RT-PCR (TaqMan) analysis.
Interestingly, when a panel of cDNAs derived from a
variety of human primary cell types and tissues was assessed for
the expression of hSCUBE3 by TaqMan analysis, we found that
hSCUBE3 RNA is highly enriched in primary cultured osteo-
blasts, followed by primary HUVeC and coronary smooth muscle
cells (Fig. 1A, top). On the other hand, hSCUBE3 transcript
levels were found to be absent or quite low in a variety of
normal human tissues (Fig. 1A, bottom). Because osteoblasts
are the prominent cells in bone tissues, we then determined
whether or not SCUBE3 mRNA is also highly expressed in
skeletons. Quantitative RT-PCR analysis was used to measure
the SCUBE3 mRNA levels in the long bones (i.e. humerus and
femur) and a number of non-osseous tissues from an adult
mouse. As shown in Fig. 1B, we found that expression of
SCUBE3 is indeed highly enriched in native bone tissue, but
low in all other non-bone tissues.

**Full-length Cloning and Sequence Analysis of hSCUBE3**—
To obtain the full-length cDNA of this gene, one pair of oligo-
nucleotides based on the public sequences was used to amplify
the entire open reading frame by PCR from cDNAs derived from
human primary osteoblasts because hSCUBE3 is highly
expressed in this cell type. This cDNA was confirmed by se-
quencing and shown to be identical to the sequence of Gen-
Bank accession number AF452494 that contains an open
reading frame of 2979 nucleotides encoding a polypeptide of
993 amino acids (see Supplemental Materials, Fig. S1). Hydro-
phobicity (6) and protein family (7) analyses predict one 22-
residue amino-terminal signal peptide, followed by nine copies of
EGF-like repeats, a spacer region, and one CUB domain (Fig.
2). Notably, there are five potential sites for N-glycosylation
located within the spacer region, between the multiple EGF-
like repeats and the CUB domain (Supplemental Materials,
Fig. S1). This domain organization is similar to that of
prior family members, SCUBE1 and SCUBE2 (2, 4, 5), and
appears unique to the SCUBE3 protein family. Mature
SCUBE3 protein is predicted (after cleavage of the signal
sequence) to be 269 amino acids long (GenBank accession
number AF452494), homologous to SCUBE1 and SCUBE2.

**Flow Cytometry Analysis**—Transfected cells were collected and sus-
pended in phosphate-buffered saline containing 2% FBS in a volume of
0.25 ml. A total of 1 μl of purified anti-FLAG M2 antibody and fluoro-
rescein isothiocyanate-conjugated goat anti-mouse secondary antibody
(1:100 dilution, Jackson ImmunoResearch Laboratories) were added
sequentially; each was incubated for 45 min on ice. FACS analyses
were performed with a FACSScan (Clontech).

**Northern Blot Analysis**—Total RNAs (10 μg) from human oste-
oblasts and human small intervertebral discs were separated on 1.2%
denaturing formaldehyde-agarose gel, transferred to nylon membranes, and
hybridized overnight with a radiolabeled 525-bp hSCUBE3 cDNA
probe (amino acids 356–531). In addition, one human tissue Northern
blot (containing 1 μg of poly(A)-enriched mRNA from a variety of human tissues) was purchased from Clontech and hybridized with a
radioiodinated hSCUBE3 cDNA probe according to the manufacturer’s
instructions. After hybridization overnight, the blots were washed once
at room temperature (25 °C) and twice at 65 °C with 0.1 × SSC (15 mM
NaCl, 1.5 mM sodium citrate) and 0.1% SDS (each wash for 1 h).

**Autoradiography** was performed at ~80 °C for 1 day. The blot was then
stripped and hybridized with a β-actin probe as a positive control.

**Immunoprecipitation and Western Blot Analysis**—Transfected cells were
harvested with phosphate-buffered saline and lysed for 1 h on ice in 0.5 ml of lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1
mM EDTA, 1 mm EGTA, 1% Triton X-100, 25 mM sodium pyrophos-
phate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin).
Lysates were centrifuged at 4 °C for 15 min at 10,000 × g. Cells lysates were incubated with 1 μg of the indicated antibody and
20 μl of protein A-agarose (Sigma) for 2 h with gentle agitation.

After three washes with lysis buffer, precipitated complexes were sol-
ubilized by boiling in Laemmli sample buffer, fractionated by SDS-
PAGE, and transferred to polyvinylidene difluoride membranes.

The membranes were blocked with phosphate-buffered saline (pH 7.5) con-
taining 0.1% gelatin and 0.05% Tween 20 and were blotted with the indicated antibodies. After two washes, the blots were incubated with
horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson Im-
munoresearch Laboratories) for 1 h. After washing the membranes, the
reactive bands were visualized with the enhanced chemiluminescence
system (Amersham Biosciences).
Normalization was performed using a variety of human primary cell types (Fig. S1). Because hSCUBE3 has five putative N-linked glycosylation sites (Fig. S1), it is likely that the recombinant hSCUBE3 protein is a secreted and cell-associated protein.

The molecular mass of the recombinant SCUBE3 protein expressed in HEK-293T cells is approximately 130 kDa, slightly larger than the predicted size of mature hSCUBE3 (Fig. S1). Because hSCUBE3 has five putative N-linked glycosylation sites (Fig. S1), it is likely that the recombinant hSCUBE3 protein is subject to a post-translational modification by glycosylation. Thus, we examined whether tunicamycin, an inhibitor of N-linked glycosylation, affected the molecular mass of the expressed protein. As shown in Fig. 4B, tunicamycin treatment of cells results in a reduction of molecular size to that of the predicted, precursor form of hSCUBE3 at about 110 kDa. These results clearly demonstrate that the majority of hSCUBE3 is N-glycosylated when expressed in HEK-293T cells.

Fig. 2. Domain structure of human SCUBE3. The region marked with the solid box indicates the putative signal peptide. The graphic shows the domain organization of human SCUBE3 protein. An apparent "spacer region" is located between the 9th EGF-like repeat and the CUB domain. Two deletion constructs are also depicted. SP, signal peptide; FL, full-length; D1 (amino acids 1–993), deletion mutant 1 (amino acids 1–803); D2, deletion mutant 2 (amino acids 1–529); E, EGF-like repeats; CUB, CUB domain.

Fig. S2). The spacer regions are the most divergent and are only about 46–50% homologous among the family members (Supplemental Materials, Fig. S3).

Northern Blot Analysis for the Expression of the hSCUBE3 Transcript—To further validate the expression of SCUBE3, Northern blots containing total RNAs (10 µg) from human primary cells or poly(A)-enriched mRNA (1 µg) from a variety of human adult tissues was hybridized with a hSCUBE3 cDNA radiolabeled probe, respectively. As shown in Fig. 3A, the expression level of hSCUBE3 was highest in human primary osteoblasts with a predominant transcript at the size of ~8.0 kb, followed by a low expression level in HUVEC with an mRNA species of about 4.0 kb. On the other hand, the tissue Northern blot analysis showed that the expression of hSCUBE3 is only barely detectable in heart, but not in all other tissues examined (Fig. 3B).

Secretion, N-Glycosylation, and Cell-surface Expression of Recombinant hSCUBE3 Protein in HEK-293T Cells—Because hSCUBE3 possesses a putative signal peptide indicative for a secreted protein and because multiple EGF-like repeats are found in a number of extracellular matrix proteins (ECM) (8, 9), we investigated whether the hSCUBE3 protein is a secretory and/or ECM protein. To this end, the recombinant hSCUBE3 protein was expressed by means of a transient expression system in HEK-293T cells. The FLAG epitope was added immediately after the signal peptide cleavage site (i.e. at the amino terminus of the processed, mature protein) for the detection of the recombinant protein (Flag.hSCUBE3). Two days after transfection and under serum-free conditions, the culture supernatants were collected, and cells were detached from dishes by EDTA treatment, and residual ECM proteins were extracted with Laemmli buffer. Samples from these three fractions were subjected to Western blot analyses using anti-FLAG antibody. As shown in Fig. 4A, like hSCUBE1, the hSCUBE3 protein was detected in the serum-free conditioned culture medium (Medium) and in cell lysates (Cell), but was not detected in the ECM fraction (Matrix) or in fractions derived from the control vector-transfected cells. These data demonstrate that the hSCUBE3 protein is a secreted and cell-associated protein.

FIG. 1. Cell type and tissue expression profile of SCUBE3 determined by real-time quantitative RT-PCR (TaqMan). A, osteoblast-enriched expression of SCUBE3. Expression of human SCUBE3 was measured by TaqMan analysis in a panel of cDNAs derived from a variety of human primary cell types (top) or human tissues (bottom). Normalization was performed using β2-microglobulin mRNA levels as control in the same reaction as described under “Experimental Procedures.” B, bone-enriched expression of SCUBE3. Expression of mouse SCUBE3 was determined by TaqMan analysis in a panel of cDNA derived from mouse long bones (humerus and femur) and a variety of non-osseous tissues. PBL, peripheral blood leukocytes; SMC, smooth muscle cells.

SCUBE3 shares an overall 66 and 60% homology with that of SCUBE1 and SCUBE2, respectively (see Supplemental Materials, Figs. S2 and S3). The CUB domain and the very carboxyterminal sequences are the most conserved regions sharing 82–90% identity in this family (Supplemental Materials, Fig. S3), followed by the amino-terminal nine copies of EGF-like repeats sharing 66–77% homology (Supplemental Materials,
Because the majority of expressed hSCUBE3 protein appears cell-associated (Fig. 4A) and because hSCUBE1 is a membrane-anchored protein (2), we next determined whether or not hSCUBE3 is capable of targeting to the cell surface by means of flow cytometric analysis. Expression plasmid encoding the Flag.hSCUBE3 was transfected into HEK-293T cells. Forty-eight hours post-transfection, cells were collected and incubated with anti-FLAG M2 antibody followed by fluorescein isothiocyanate-conjugated goat anti-mouse IgG secondary antibody, then analyzed by flow cytometry. As shown in Fig. 4C, expression of the Flag.hSCUBE3 protein resulted in a shift of a population of fluorescein isothiocyanate-labeled cells by fluorescence-activated cell sorter analysis, suggesting that hSCUBE3, like hSCUBE1, is targeted to the cell surface.

Homo- and Hetero-oligomerization of hSCUBE3—We have previously shown that SCUBE1 and SCUBE2 are capable of forming a homomeric or heteromeric complex when overexpressed in HEK-293 cells. To ascertain whether or not this newly identified family member, SCUBE3, can form the oligomeric complexes, the differentially epitope-tagged SCUBE3 and SCUBE1 expression plasmids were singly or co-transfected into HEK-293T cells. Two days after transfection, cell lysates were immunoprecipitated followed by Western blot analysis to determine the protein associations. When SCUBE3.Myc is coexpressed with either Flag.SCUBE1 or Flag.SCUBE3, immunoprecipitation with anti-Myc antibody results in the co-immunoprecipitation of either protein, respectively (Fig. 5). Likewise, the reciprocal immunoprecipitation of anti-FLAG antibody followed by Western blotting with anti-Myc antibody confirmed the formation of homo- and hetero-oligomeric complexes between SCUBE3 and SCUBE1, at least in HEK-293T cells.

Proteolytic Processing of the Secreted SCUBE3 Protein—Because two novel CUB domain-containing platelet-derived growth factor family members, platelet-derived growth factor-C and -D (10–13), require proteolytic activation by an as yet unidentified protease in FBS, we tested whether the secreted hSCUBE3 protein can be subject to proteolytic cleavage in the presence of FBS. After transfection with the Flag.hSCUBE3 expression plasmid, cells were cultured under serum-free or 10% FBS/Dulbecco’s modified Eagle’s medium, respectively. Forty-eight hours later, the conditioned culture medium was immunoprecipitated followed by immunoblotting with anti-FLAG antibody. As shown in Fig. 6A, under serum-free conditions, the molecular mass of the secreted hSCUBE3 protein is ~130 kDa, in accordance with that of cell-associated full-length hSCUBE3 (Fig. 4A). However, in the presence of FBS, a fraction of the secreted protein undergoes limited proteolysis, resulting in a processed product carrying the molecular mass of approximate 65 kDa (Fig. 6A). These results suggest that the secreted hSCUBE3 protein, like platelet-derived growth factor-C and -D, can be proteolytically cleaved by a protease present in FBS.

To further map the potential proteolytic cleavage site, two carboxyl-terminus deletion mutants, D1 (lacking the CUB domain only) and D2 (further lacking part of the spacer region) (Fig. 2), were generated and compared with the processed hSCUBE3 product. As shown in Fig. 6B, the hSCUBE3-D2 protein migrated at the similar molecular mass as that seen in the processed hSCUBE3 protein. These data strongly suggest that the potential proteolytic cleavage site is located within the spacer region, near the boundary of deletion mutant D2.

DISCUSSION

In this report, we have identified and characterized one additional member of the human SCUBE protein family, named SCUBE3. Human SCUBE3 (hSCUBE3) possesses a unique domain organization belonging to the SCUBE family, which consists of one signal peptide, one CUB domain, and multiple copies of EGF-like repeats (Fig. 2). In addition, an apparent spacer region containing five potential N-glycosylation sites is located between the amino-terminal cluster of EGF-like repeats and the carboxyl-terminal CUB domain (see Supplementary Materials, Fig. S1). This region has been shown
to play a critical role for the secretion and cell-surface expression of SCUBE1 (2). It remains to be determined whether or not the spacer region in hSCUBE3 functions in a similar way.

The EGF-like repeat is a six-cysteine conserved motif found in a number of proteins such as secreted growth factors, adhesion molecules, signaling proteins, transmembrane receptors, and components of the ECM (9). Previous reports suggested the presence of the 10th motif of EGF-like repeat in SCUBE1 and SCUBE2 proteins (2, 4, 5). The corresponding region (amino

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**FIG. 4.** Secretion, glycosylation, and cell-surface expression of SCUBE3 in HEK-293T cells. A, SCUBE3 is a secreted and cell-associated protein. HEK-293T cells were transfected with empty vector or expression vectors encoding FLAG-tagged human SCUBE3 (Flag-hSCUBE3) or SCUBE1 (Flag.hSCUBE1) as positive control (2). Forty-eight post-transfection, the serum-free conditioned medium was collected, and cells were detached with phosphate-buffered saline/EDTA. The extracellular matrix on the culture dish was extracted with Laemmli sample buffer. Samples from the serum-free conditioned culture medium (Medium), cell lysates (Cell), and the extracellular matrix (Matrix) were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Recombinant SCUBE proteins were detected by Western blotting with anti-FLAG M2 antibody. B, N-glycosylation of human SCUBE3. HEK-293T cells were transfected with empty vector or expression plasmid encoding Flag.hSCUBE3 protein. Transfected cells were cultured in the absence (−) or presence (+) of tunicamycin for 24 h. Cell lysates from each culture were analyzed by Western blotting with anti-FLAG antibody. C, cell-surface expression of human SCUBE3 protein. HEK-293T cells were transfected with empty vector or expression vector encoding Flag.hSCUBE3 or Flag.hSCUBE1 proteins, respectively. Forty-eight hours post-transfection, transfected cells were detached and stained with anti-FLAG antibody and subjected to flow cytometry analysis as described under “Experimental Procedures.”

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**FIG. 5.** Homo- and hetero-oligomerization of human SCUBE3 in transfected HEK-293T cells. HEK-293T cells were transfected with Myc-tagged SCUBE3 (hSCUBE3.Myc) singly or co-transfected with the Flag.hSCUBE1 or Flag.hSCUBE3 as indicated. The total amount of DNA was kept constant in all transfections by supplementing the empty vector DNA. Two days later, detergent lysates of each transfection were subjected to immunoprecipitate (IP) by FLAG or Myc antibodies, followed by immunoblotting (WB) using FLAG and Myc antibodies, respectively, to determine the associated proteins. Cell lysates were also immunoblotted with anti-FLAG or Myc antibodies to examine the protein expression levels (bottom two panels).

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**FIG. 6.** Limited proteolysis of the secreted SCUBE3 protein in the presence of FBS. A, the secreted SCUBE3 protein is proteolytically cleaved in the presence of FBS. HEK-293T cells were transfected with the control vector and plasmid encoding the Flag-hSCUBE3. After transfection, the cells were then cultured with serum-free Dulbecco’s modified Eagle’s medium or in Dulbecco’s modified Eagle’s medium containing 10% FBS. Forty-eight hours later, the conditioned cultured medium was immunoprecipitated with anti-FLAG antibody, separated by SDS-PAGE, and analyzed by Western blotting using anti-FLAG antibody. B, proteolytic cleavage within the spacer region of the secreted hSCUBE3. HEK-293T cells were transfected with various expression plasmids encoding the Flag.hSCUBE3 full-length (FL), deletion mutants (D1 and D2), and the control vector. Two days after transfection, the cell lysates (cell) and the culture supernatant (medium) were immunoprecipitated and immunoblotted with anti-FLAG antibody. The positions of the SCUBE3 full-length (arrow) and its proteolytically cleaved proteins (arrowhead) are indicted.
acids 742–779) in hSCUBE3 lacks one of six invariant cysteines essential for maintaining the intermolecular disulfide bonds, therefore, this region is not presented as an authentic EGF-like repeat in Fig. 2. Further motif analysis of SCUBE3 utilizing the SMART protein analysis tool (14) revealed that six of nine EGF-like repeats (i.e., repeats 1, 2, 3, 7, 8, and 9) contain multiple conserved amino acid residues essential for the calcium-binding capacity (15), indicating that SCUBE3 may exert its biological functions in a calcium-dependent manner.

Based on the quantitative TaqMan and Northern blot analyses, we found that hSCUBE3 is highly selective and enriched in human osteoblasts as a primary mRNA species of ~8.0 kb (Figs. 1A and 3). Consistent with the osteoblast-enriched expression, SCUBE3 mRNA was indeed highly expressed in the long bone samples derived from humerus and femur in adult mouse, but virtually undetectable in all other non-osseous tissues (Fig. 1B). Thus, SCUBE3 appears to be an osteoblast- and bone-enriched gene. In addition, hSCUBE3 is expressed at relatively low levels in HUVeC and heart with a smaller 4.0-kb transcript (Fig. 3). Interestingly, the endothelial cells appear to be a common cell type expressed by all three members of the SCUBE family (2). Two distinct transcripts of 8.0 and 4.0 kb may represent the splice variants of hSCUBE3 mRNA in different cell types. Consistently, two splice variants of SCUBE1 and SCUBE2 had been described (4, 5). In addition, the larger transcript of 8.0 kb may be attributed to an extended 3′-untranslated region, because additional 2.1-kb sequences were found in the longest public cDNA clone (GenBank™ accession number AF452494). Likewise, a number of public cDNA clones representing mouse Scube3 also assemble into a contig with an extended 3.6 kb of the 3′-untranslated region.

Utilizing an approach of gene prediction, we also identified the apparent homologue of SCUBE3 in the mouse genome (www.ensembl.org). Mouse Scube3 shares an overall 96% protein identity with hSCUBE3 and is located on mouse chromosome 17.B region, syntenic to human 6p21.3 where the SCUBE3 gene resides. As a candidate approach, we searched the human disease data base (the Online Mendelian Inheritance in Man) for bone or bone-related diseases in the 6p21 region. Paget’s disease of bone (PDB) is a metabolic bone disease composed of a genetically and clinically heterogeneous group of patients who experience bone pain, enlargement, and deformities at the pagetic site (16). Some cases are caused by mutations in the RANK gene on 18q21 (PDB2; OMIM number 601530) (17), whereas mutations in the SQSTM1 gene, encoding the ubiquitin-binding protein sequestosome 1 (p62, ref. 18), is associated with the RANK-induced NF-κB signaling pathway, were found to be the cause of Paget’s disease in families with linkage to 5q31. It is well documented that both physical and biochemical communications exist between blood vessel-endothelial cells and bone-forming osteoblasts through a number of secreted factors, like fibroblast growth factors and transforming growth factor-β, during bone formation and repair (19). It remains to be determined whether or not the endothelial SCUBE1 and osteoblast-enriched SCUBE3 indeed interact in vivo and play a role in the cell-cell communications between these two cell types.

It is interesting to find that an as yet unidentified protease present in normal FBS is essential to proteolytically process the secreted SCUBE3, which in turn releases the multiple EGF-like repeats from the CUB domain. However, it is unclear whether or not this proteolytic cleavage represents an activation mechanism as described for the regulation of the CUB domain-containing plasminogen-derived growth factor-C and -D (10–13). Close examination of the margin sequence in the carboxyl-terminal deletion mutant D2 revealed a minimal recognition site (RXXR, residues 537–540) for the furin-like protease (20) within the spacer region of the hSCUBE3 protein (Supplemental Materials, Fig. S3). Because the processed hSCUBE3 protein migrated at the similar position to that of the deletion mutant D2 (Fig. 6B), it is possible that the secreted hSCUBE3 protein is proteolytically processed within the spacer region by a furin-like protease. Nevertheless, it remains to be established whether the serum-derived protease activity or other cellular protease(s) are of physiological relevance.

Bone formation and resorption are often described as two separate, independent processes; however, in healthy skeleton they are tightly coupled within temporary anatomic structures, known as the basic multicellular unit (21). The basic multicellular unit consists of a group of osteoclasts in the front, a team of osteoblasts in the rear, a central vascular capillary and associated connective tissue (21). High expression of a novel secreted, cell-surface protein, hSCUBE3, in human osteoblasts suggests that this protein may play an important role in skeletal biology through acting on cell types within the basic multicellular unit, such as osteoblasts, osteoclasts, or endothelial cells, in an autocrine/paracrine or endocrine fashion.

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