DOMAIN AND FUNCTIONAL ANALYSIS OF A NOVEL PLATELET-ENDOTHELIAL CELL SURFACE PROTEIN, SCUBE1*
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SCUBE1 (signal peptide-CUB-EGF domain-containing protein 1) is a novel, secreted, cell-surface glycoprotein expressed during early embryogenesis and found in platelet and endothelial cells. This protein is composed of an N-terminal signal peptide sequence followed by 9 tandemly arranged epidermal growth factor (EGF)-like repeats, a spacer region, 3 cysteine-rich repeat motifs and one CUB domain at the C terminus. However, little is known about its domain and biological function. Here, we generated a comprehensive panel of domain deletion constructs and a new genetic mouse model with targeted disruption of Scube1(SCube1Δcub/Δcub) to investigate the domain function and biological significance. A number of cell-based assays were utilized to define the critical role of the spacer region for membrane association and establish that the EGF-like repeats 7-9 are sufficient for the formation of SCUBE1-mediated homophilic adhesions in a calcium-dependent fashion. Biochemical and molecular analyses showed that the C-terminal cysteine-rich motifs and CUB domain could directly bind and antagonize the bone morphogenetic protein (BMP) activity. Furthermore, genetic ablation of this C-terminal region resulted in brain malformation in the Scube1Δcub/Δcub embryos. Together, our results support the dual roles of SCUBE1 on brain morphogenesis and cell-cell adhesions through its distinct domain function.

SCUBE1 (signal peptide-CUB-EGF-like repeat-containing protein 1) is the founding member of an evolutionarily conserved SCUBE gene family (1,2). To date, three distinct isoforms have been cloned and named SCUBE1 to SCUBE3 according to their order of discovery in the mammal (1-4). These genes coding for polypeptide molecules of about 1000 amino acids share an organized protein domain structure of at least five recognizable motifs: an N-terminal signal peptide sequence, 9 tandem repeats of epidermal growth factor (EGF)-like modules, a large N-glycosylated spacer region followed by three repeated stretches of 6-cysteine residues with unique and regular spacing, and one CUB...
domain at the C-terminus (see Fig. 1A). Our previous study demonstrated that the signal peptide sequence is sufficient to direct the expressed SCUBE1 protein into the secretory pathway and results in a secreted and surface-associated protein (1). However, little is known about the functional significance of the other 4 domains in SCUBE1.

SCUBE genes have been shown to be expressed predominantly in a variety of developing tissues, including gonads, the central nervous system, dermomyotome, digital mesenchyme, and limb buds during mouse embryogenesis (2,3,5), which implies that the proteins may play important roles in development. However, direct functional studies of the roles of the SCUBE genes during mammalian development are still lacking. In addition to its embryonic expression, SCUBE1 was found to be expressed in the endothelium and platelets (1,6). Our recent study showed that SCUBE1 is stored in the platelet α-granules and exposed to the cell surface upon platelet stimulation and activation (6). Yet, the molecular function for such targeted exposure of SCUBE1 on the cell surface has not been addressed.

Here, we investigated the domain and biological functions of SCUBE1 by utilizing a series of SCUBE1 domain-specific deletion constructs and employing a new genetic mouse model with targeted disruption of the Scube1 gene. Our results demonstrated, for the first time, that the N-terminal EGF-like repeats participate in the formation of adhesions in a Ca\(^{2+}\)-dependent fashion and the EGF-like repeats 7-9 are sufficient for reciprocal and lateral interactions between SCUBE1 in homophilic adhesions. Moreover, gene-targeting study indicated that SCUBE1 is required during the early stages of central nervous system development, possibly through modulating the activity of members of the bone morphogenetic protein (BMP) family of proteins via its C-terminal 6-cysteine repeat motifs and the CUB domain.

**EXPERIMENTAL PROCEDURES**

*Cell culture, construction of expression plasmids, and transfection* - A2058 melanoma cells, human embryonic kidney (HEK)-293T and GP2-293 retrovirus packaging cells were maintained in Dulbecco’s minimal essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin at 37 °C in an atmosphere of 5% CO\(_2\). The epitope-tagged version of the SCUBE1 mutant was constructed as described (1). The transfection involved use of Lipofectamine 2000 (Invitrogen).

*Immunoprecipitation and western blot analysis* - Two days after transfection, cell lysates were clarified by centrifugation at 10,000 xg for 20 min at 4 °C. Samples underwent immunoprecipitation, followed by western blot analysis as described (1).

*Cell aggregation assay* - A2058 stable cells were detached by trypsin (0.01%-EDTA (2.5 mM) treatment and suspended in DMEM medium, either containing 5 mM CaCl\(_2\) or Ca\(^{2+}\) free, at 1 x 10\(^6\) cells/ml in polystyrene tubes. Then, tubes...
were incubated on a rotating platform (10 rpm) at 37 °C for 1 h. The extent of cell aggregation was viewed microscopically and photographed. For quantitation, cell clusters of more than four cells were counted as being aggregated. The data are shown as the means ± SEM of three independent experiments in duplicate.

Luciferase activity assays - Human HepG2 cells (3 x 10^5 cells per well) were seeded into 24-well plates and transfected on the following day with 0.4 μg of the BMP-inducible luciferase reporter I-BRE-Luc and 0.01 μg of the Renilla luciferase reporter vector used as an internal control. The transfected responding cells (HepG2) were stimulated with conditioned media derived from the signaling cells (HEK-293T) transfected with the BMP2 expression plasmid alone or in a combination of various SCUBE1 deletion constructs. Luciferase activity was measured following 24-h incubation by the use of the dual reporter system (Promega). Data are expressed as relative luciferase activity (firefly luciferase activity divided by Renilla luciferase activity).

Targeted disruption of the Scube1 gene in mice - The targeting vector (MHPP-84m2) obtained from the MICER website (http://www.sanger.ac.uk/micer/) was digested with ClaI (Fig. 8A). The ES cell line R1, established from hybrid of two 129 substrain (129X1/SvJ and 129S1) F1 male blastocyst (7), was transfected with linearized targeting vector, and selection involved use of puromycin. Of a total of 251 ES cell clones, we obtained 13 correctly targeted ES clones verified by Southern blot and/or PCR analysis. The correctly targeted ES cell clones were injected into blastocysts of C57BL/6 mice. The targeted allele was backcrossed with C57BL/6 females for at least three generations before phenotyping experiments.

Histological and immunohistochemical analysis - Embryos were dissected from pregnant mice at the indicated time, fixed and embedded in paraffin. Sections were cut at 5 μm and stained with hematoxylin and eosin. For SCUBE1 immunohistology, sections were probed with a monoclonal antibody specifically against the CUB domain of SCUBE1 (6).

Proliferation and apoptosis assays - Sections were stained with rabbit anti-mouse Ki-67 antibody (DakoCytomation) for active cell proliferation and counterstained with hematoxylin to detect all nuclei. Detection of apoptotic cells involved the terminal dUTP nick-end labeling (TUNEL) reaction kit (Roche) and counterstaining by DAPI.

Statistical analysis - Data are expressed as mean ± SEM. Differences between groups were analyzed by unpaired Student’s t-test. P < 0.05 was considered significant.

RESULTS

Molecular dissection of the SCUBE1 domain responsible for secretion and cell-surface association - We previously showed that overexpression of SCUBE1 in HEK-293T cells
resulted in a secreted glycoprotein tethered on the cell surface (1). However, the structure responsible for the surface targeting and membrane association was not precisely defined. To delineate the domain required for the secretion and cell surface anchorage, we then generated a comprehensive panel of mutated SCUBE1 expression constructs (D1 to D7) with specific deletions of the EGF-like repeats, spacer region, cysteine-rich repeats, or CUB domain (Fig. 1A). The Flag epitope tag was added at the amino terminus for easy detection of these mutated SCUBE1 forms.

The SCUBE1 deletion proteins were expressed by means of transient expression in HEK-293T cells. Two days after transfection, the conditioned media and cell lysates were collected and subjected to western blot analysis with anti-Flag antibody. As shown in Fig. 1B, all SCUBE1 deletion constructs produced corresponding recombinant proteins in HEK-293T cells (bottom panel). Furthermore, immunoblotting of the conditioned media from transfected cells showed an effective secretion of all expression products, except for the SCUBE1-D2 mutant protein, which lacks the spacer region (Fig. 1B, top panel). Adding back this region restored the secretion of the D2 mutant into the conditioned culture medium (data not shown). Together, these data suggest that the spacer region, located between the EGF-like domains and the cysteine-rich repeats, is capable of directing the expressed SCUBE1 protein through the secretory pathway for secretion, at least when overexpressed in HEK-293T cells.

The same set of transfected cells was detached and stained with anti-Flag antibody to determine their surface expression by flow cytometry (Fig. 1C). Flow cytometrical analysis showed SCUBE1-D2, -D4, -D5, and -D6 deletion mutants defective in membrane association, but SCUBE1-FL, -D1, -D3, and -D7 mutant protein, which all contain the spacer region, was expressed on the cell surface. Together, our results support the concept that the spacer region has at least two functions: to target SCUBE1 for secretion and anchor the molecule on the cell surface.

Establishment of cell lines expressing a series of SCUBE1 EGF-like repeat deletion mutants - Our previous study showed that the preformed SCUBE1 protein stored in the platelet α-granules could be translocated to the surface upon platelet activation (6). However, the functional significance of such targeted exposure of SCUBE1 on the cell surface remained elusive. Because certain EGF-like repeats mediate homophilic or heterophilic protein-protein interactions (8,9), we next examined by suspension cell aggregation assay whether the amino terminal EGF-like repeats of SCUBE1 play a role on cell-cell interaction.

To further evaluate the relative contribution of the EGF-like repeats in the adhesion function of SCUBE1, we first constructed four deletion mutants consisting of the EGF-like modules 1-9 (E1-9), 4-9 (E4-9), 7-9 (E7-9), or only E7-9 deletion (ΔE7-9) into a retrovirus vector, pLNCX-eB7 (10). In addition, an HA epitope tag was added at the N-terminus for easy detection, and the mutant constructs were further fused to
the B7 extracellular and transmembrane domain (B7TM) to effectively target the chimeric protein on the plasma membrane (Fig. 2A).

The melanoma A2058 cells lacking endogenous SCUBE1 expression were infected with recombinant retroviral particles and selected in G418 to obtain four independent A2058/SCUBE1-E1-9, -E4-9, -E7-9, or -ΔE7-9 stable transfectant cell lines. Western blot analysis with the anti-HA antibody demonstrated the SCUBE1 EGF-like repeat deletion mutant proteins to be produced at comparable levels with the expected molecular masses (Fig. 2B). Most importantly, flow cytometry using the anti-HA or anti-SCUBE1 antibody confirmed that all of these SCUBE1 EGF-like repeat mutants expressed by A2058 cells were transported to the cell surface (Fig. 2C).

*The EGF-like repeats 7-9 are sufficient, but not necessary, for reciprocal (trans) or lateral (cis) interactions between SCUBE1 molecules.* To examine the involvement of SCUBE1 EGF-like repeats in intercellular adhesion, we evaluated the aggregating properties of the SCUBE1 EGF-like repeat mutant stable transfectant cell lines by cell aggregation assay. Monolayer cultures of the parental (control) or transfected stable cell lines were dissociated into single cells and allowed to aggregate in suspension culture. When the suspensions were gently shaken, A2058 cells expressing SCUBE1 EGF-like repeat mutants tended to aggregate (Fig. 3). The parental A2058 cells aggregated poorly in suspension (< 3%), but a good proportion (∼8% to 20%) of the SCUBE1 EGF-like repeat mutant-transfected clones aggregated after an hour of shaking (Fig. 3).

The chelating of calcium by Ca²⁺-binding EGF-like domains is important for the structural integrity and function of several other EGF-like domain-containing proteins (11,12). Because 6 of 9 EGF-like modules (i.e., repeats 1-3 and 7-9) contain Ca²⁺-binding consensus sequences (1,6), we hypothesized that these EGF-like repeats may function in a Ca²⁺-dependent manner. When the same cell lines were dissociated with gentle pipetting in the presence of 5 mM EDTA or allowed to aggregate in the absence of Ca²⁺, no apparent aggregation was observed during one hour of incubation for either the parental or EGF-like repeat deletion transfectants (Fig. 3A).

To further address the specificity of the EGF-like repeats, the recombinant GST fusion protein fragment containing the EGF-like repeats (GST-E4-9) or spacer region (GST-Spacer) was added into the cell aggregation assay to block the cell-cell interactions. Soluble EGF-like repeat protein GST-E4-9 clearly blocked A2058-SCUBE1-E7-9-mediated cell aggregation assay, whereas GST alone or recombinant spacer region protein (GST-Spacer) could not block SCUBE1-induced cell aggregation (Fig. 4).

We previously showed that human SCUBE1 proteins are capable of forming oligomeric complexes when overexpressed in HEK-293T cells (1). To further determine the relative contribution of which EGF-like repeat modules involved in lateral (cis) intracellular oligomerization, we transiently co-expressed three deletion constructs, HA-tagged SCUBE1-E1-9, -E4-9, -E7-9, or -ΔE7-9 with Flag.SCUBE1-D2 containing only the EGF-like
repeats 1-9 in HEK-293T cells. Immunoprecipitation of HA.SCUBE1-E1-9, -E4-9, -E7-9, or -ΔE7-9 resulted in the co-precipitation of Flag.SCUBE1-D2 (Fig. 5). Therefore, the EGF-like repeats 7-9 are also sufficient, but not necessary, for homophilic lateral SCUBE1 interactions within the same cells.

Molecular analysis of the C-terminal 6-cysteine repeat motif and CUB domain - Recent genetic analysis identified that the zebrafish orthologue of the mammalian SCUBE2 gene acts upstream of hedgehog (HH) ligands or through a parallel pathway (13-15). In addition, a nonsense mutation in the ty97 allele encodes a truncated null protein lacking the cysteine-rich repeat motif and the CUB domain, implying that this C-terminal region is essential for SCUBE2 function (13-15). From this genetic evidence, the SCUBE2 protein is postulated to play a critical role for transport or stability of HH signaling in the extracellular environment (15), function as an endocytic receptor during reception of the HH signal (13), or modulate the long-range action of BMP-dependent signaling in the neural tube and somites (14), but the exact biochemical and molecular mechanism underlying the SCUBE2 action remains unclear.

To further explore the molecular function of the C-terminal region of SCUBE1, we generated one additional deletion mutant, SCUBE1-ty97 (Q623 to stop codon), mimicking the ty97 mutant allele in the zebrafish Scube2 gene by removing the 6-cysteine repeat motif and the CUB domain (Fig. 6A). Since one previous report demonstrated that BMP activity can be attenuated by the co-expression of SCUBE2 in the zebrafish (14), we next investigated whether the SCUBE1 C-terminal domain could directly interact with BMP2 protein and form a complex. HEK-293T cells were transfected with a Myc-tagged BMP2 expression plasmid alone or in combination with a series of Flag-tagged SCUBE1 domain deletion constructs (Fig. 6A). Two days after transfection, cell lysates underwent immunoprecipitation with the anti-Myc monoclonal antibody, and the precipitates were analyzed by immunoblotting with anti-Flag monoclonal antibody to determine the protein association. Immunoprecipitation with anti-Myc antibody resulted in a specific co-immunoprecipitation of the SCUBE1-D4 or -D5 deletion protein (Fig. 6B), which suggests that SCUBE1 could form a complex with BMP2 through its C-terminal CUB domain.

To further evaluate whether the interaction between SCUBE1-D4/-D5 and BMP2 affects the long-range signaling of BMP2, we co-cultured the conditioned media derived from HEK-293T cells co-transfected with BMP2 alone or in combination with SCUBE1 deletion constructs (signaling cells) added to the responding cells, HepG2 cells containing the BMP-inducible promoter luciferase reporter construct I-BRE-Luc (16) (Fig. 7A). As expected, BMP2 alone produced by the signaling cells could act as a long-range signaling molecule by inducing about a 10-fold increase in luciferase activity (Fig. 7B). Although the BMP2 proteins co-expressed with the SCUBE1-FL, -ty97, -D5, or -D6 triggered the BMP-mediated transcriptional activation equally well, co-expression with the SCUBE1-D4 mutant
resulted in a marked attenuation of the BMP response (Fig. 7B).

Since the proteolytic processing of the large prepro precursor of BMP (proBMP) and its subsequent secretion into the extracellular environment are the required steps in the formation of the biologically active BMP ligand, we next investigated whether the inhibition of BMP2 signaling by SCUBE1-D4 occurs in the intracellular or extracellular space. HEK-293T cells were transfected with plasmids expressing proBMP2 alone or in combination with various SCUBE1 deletion constructs. Western blot analysis of the cell lysates and condition medium from these cultures revealed all deletion mutants with no effect on total proBMP2 synthesis (Fig. 7C), whereas only the SCUBE1-D4 mutant potently inhibited the secretion of mature BMP2 into the culture medium.

Targeted disruption of the Scube1 gene in mice produces a neural tube closure defect over the midbrain - To further evaluate the biological function of the C-terminal domain of SCUBE1 in vivo, we disrupted the mouse Scube1 gene at the end of exon 16 by use of an insertional targeting vector obtained from a public resource (17). This vector was expected to result in the duplication of exons 11-16 and disruption of splicing to produce a mutant transcript coding for a truncated protein lacking the functional 6-cysteine repeat motifs (exons 16-19) and the CUB domain (exons 20 and 21) (Fig. 8A). The correctly targeted embryonic stem cell clones and chimeric mice carrying germline transmission of the mutant allele of Scube1 were confirmed by Southern blot (Fig. 8B) and genomic PCR analyses with one primer pair designed from the vector backbone and the other designed from outside the targeting construct (Fig. 8A). As well, the expression of the mutant transcript in hetero- or homozygous mutant mice was confirmed by reverse transcriptase PCR analysis and direct sequencing of the PCR product (data not shown). Most importantly, the Scube1\textsuperscript{Δcub/Δcub} mutant mice lacked the expression of the functional C-terminal region because SCUBE1 immunoreactivity was not seen in the mutant brain sections stained with a monoclonal antibody specific against the CUB domain (6) (Fig. 8C). However, immunohistchemistry stained with the N-terminus specific monoclonal antibody could still detected the immunoreactivity in the mutant neural tissues including forebrain (f), hindbrain (h), and cranial ganglia (asterisks), suggesting the targeted allele is not a true null allele (see Fig. S1). Instead, a truncated form of SCUBE1 containing the N-terminal fragment is expressed in the mutant animal, as the protein fragment can be detected using an antibody specific for the N-terminus. Thus, this allele is designated as Scube1\textsuperscript{Δcub/Δcub} on the basis of the lack of the CUB immunoreactivity (Fig. 8C).

Mice heterozygous for the Scube1 mutation have a wild-type phenotype and normal fertility. However, the Scube1\textsuperscript{Δcub/Δcub} newborn pups died shortly after birth and manifested a phenotypic defect showing acrania and lack of brain tissue (Fig. 9A). In addition, mutant embryos recovered from E12.5 to E17.5 were alive (determined by active heart beating and/or limb movement) with exposed and progressively degenerating brain
tissue (Fig. 10B and data not shown).

The acrania phenotype was more clearly revealed by staining E14.5 skeletons with alcian blue (cartilage) and alizarin red (ossified bone) (Fig.s 9B and 9C). Despite no apparent defects in the limb and skeletal structures, the cranial vault is missing in the Scube1Δcub/Δcub skulls (Fig.s 9B and 9C). The interparietal and supraoccipital bones were missing, and only a small portion of the frontal and parietal bones were present at their lateral basal levels. Thus, the defects in cranial bone formation occurred early in development.

Close examination and histological analyses of E12.5 Scube1Δcub/Δcub embryos revealed that the brain malformation between the diencephalon and myelencephalon (Fig. 10B, between arrows), which resulted in exencephaly restricted to the midbrain because of overproliferation of neural tissue and everted cranial neural folds (Fig. 10D, asterisks). Further histological sections at the level of the eye uncovered distinct pattern abnormalities, including thickening of the forebrain neuroepithelium wall (Fig. 10F) and hyperplasty in the cranial ganglia, especially the trigeminal and vestibulocochlear ganglia (gV or gVIII in Fig. 8C). However, the hindbrain neural tube seemed less affected (Fig. 8C).

Given the increase in forebrain neuroepithelial wall thickness in Scube1Δcub/Δcub embryos (Fig. 10F), we then analyzed the mitotic activity in this tissue. Interestingly, increased proliferation rate was observed in the forebrain neural tube, as evidenced by the number of nuclei stained positive for Ki-67, a cell proliferation marker (61.3 ± 2.9 % for Scube1Δcub/Δcub vs. 48.5 ± 2.5 % for the wild-type; p < 0.05; n = 3 animals for each genotype). However, the proliferation rate of the hindbrain neural tube was not affected (11.1 ± 3.1 % for Scube1Δcub/Δcub versus 12.3 ± 2.0 % for the wild-type; p > 0.05). Furthermore, TUNEL assay did not reveal any discernable difference in the number of apoptotic cells at E12.5 in the forebrain or hindbrain neuroepithelium (data not shown).

DISCUSSION

In the present study, we analyzed the domain and the biological function of SCUBE1, the first member of a small secreted, cell-surface SCUBE protein family. In vitro cell-based assays and in vivo gene ablation experiments support the dual roles of the Scube1 gene product during early embryonic development and postnatal cell-cell adhesions. In addition, our results suggest that the SCUBE1 protein is organized in a modular fashion and has a unique function associated with each domain. For example, it not only could function as an antagonist for the BMP signaling through its C-terminal domain but may also act as a cell adhesion molecule through Ca²⁺-dependent interactions mediated by EGF-like repeat modules.

Moreover, our mapping of the secretion and membrane-association domain to the spacer region allowed us to further assign one additional specific function to a structural domain of SCUBE1 (Fig. 1). Because targeting of some proteins to the cell membrane takes place through posttranslational modifications by N-glycosylation or glycosylphosphatidylinositol (GPI) linkage (18-20), we examined whether
these modifications underscore the membrane association by the spacer region. However, flow cytometry and western blot analysis confirmed that neither site-directed mutagenesis of the only glycosylation site (N466Q) residing within the spacer region nor treatment of transfected cells with phosphotidylinositol-specific phospholipase C affected the surface expression of SCUBE1-D7 mutant protein in HEK-293T cells (data not shown). Thus, N-linked glycosylation or GPI linkage is unlikely to be involved in the spacer region-mediated cell surface anchorage of SCUBE1. Although the spacer region contains no classical transmembrane segment, helical plotting analysis identified an amphipathic α-helix (residues 567-589) similar to the membrane-associated, lipid-embedded amphipathic helix structures described in apolipoproteins (21) and Cubilin (22). However, whether this amphipathic α-helix or other mechanisms contribute to the membrane anchorage of SCUBE1 requires further investigation.

In addition, although the Scube1 gene is expressed in various embryonic tissues in the mouse, including the ventral forebrain, limb buds, somites, and developing gonads (2), we observed abnormality only in the ventral forebrain development in Scube1Δcub/Δcub mice. A simple explanation for the requirement of SCUBE1 in only the ventral forebrain development is that other SCUBE members or factors with redundant function might compensate for SCUBE1 in other regions of the Scube1Δcub/Δcub embryo.

For the four EGF-like repeat deletion constructs tested, we consistently detected reduced aggregation between the A2058-SCUBE1-E1-9 cells as compared with A2058-SCUBE1-E4-9 or -E7-9 cells (Fig. 3). One explanation for this reduced aggregation could be that the SCUBE1-E1-9 construct simply does not express as much protein at the cell surface as the SCUBE1-E4-9 or -E7-9 construct (Fig. 2C), thereby diminishing the strength of the interaction. Alternatively, the difference in strength of interaction may indicate a fundamental functional difference between SCUBE1-E1-9 and SCUBE1-E4-9/-E7-9. For example, the first 3 EGF-like repeats 1-3 may negatively regulate the cell adhesive function of SCUBE1 mediated by the EGF-like repeats 7-9 in vivo. Clearly, additional studies are needed to clarify this issue.

In addition, our deletion analysis demonstrated that the EGF-like repeats 7-9 appear to function as an independent molecular unit that is sufficient for the formation of SCUBE1-mediated homophilic adhesions between adjacent cells, even in the absence of most of the EGF-like repeats (Fig. 3). However, the A2058 cells expressing the deletion mutant lacking only the EGF-like repeats 7-9 (ΔE7-9) could still form significant numbers of aggregates as compared to the parental cells (Fig. 3). Together, these results reveal that the EGF-like repeats 7-9 of SCUBE1 are sufficient, but not necessary, for trans-interactions between SCUBE1 molecules on adjacent cells in a Ca2+-dependent fashion. In accordance with our previous study (6), these findings further support the adhesive and pathological role for the surface-exposed platelet SCUBE1 in mediating the platelet-platelet agglutination under
thrombotic conditions.

The EGF-like motif is characterized by a conserved arrangement of 6-cysteine residues that form three pairs of disulfide bonds (23). Furthermore, most of these repeats (6 out of 9, repeats 1-3 and 7-9) in SCUBE1 have calcium-binding consensus sequences: D/N - x - D/N - E/Q - x_m - D/N^* - Y/F (where m and n are variable and * indicates possible β-hydroxylation) (23). The Ca^{2+}-binding EGF-like repeat is a common structural module used by cell-surface proteins and can be found in extracellular proteins involved in mediating protein-protein and protein-carbohydrate binding interactions (23,24). They are also important in functionally diverse processes, such as cell adhesion, receptor-ligand interaction, cell fate decision, blood coagulation, cholesterol uptake, and maintenance of extracellular matrix architecture (11).

Although we have not yet demonstrated that these EGF-like repeats actually bind Ca^{2+} ions, the Ca^{2+}-dependent nature of the SCUBE1 EGF-like repeat-mediated reciprocal interactions is consistent with the role of Ca^{2+} in the biological functions of other EGF-like repeat-containing proteins (11). Nuclear magnetic resonance studies have shown that the Ca^{2+} ion plays a key role in maintaining the conformational rigidity and binding surfaces of calcium-binding EGF-like repeats (25,26). However, whether the calcium-binding EGF-like repeats in SCUBE1 also undergo a conformational rearrangement upon calcium binding and how these Ca^{2+}-induced conformational changes account for the homophilic reciprocal interactions between SCUBE1 proteins require further investigation.

The CUB domain is a 110 amino acid-long, widespread module found in developmentally regulated proteins (27) and is known to form a β-barrel (28). The acronym CUB (Complement C1r/C1s, Uegf, and Bmp1) is derived from the first letters of the initial founding members of this protein group (27). The CUB domains have been implicated in protein-protein and protein-carbohydrate interactions (27) and may confer specificity to ligand binding; for example, different CUB domains in Cubilin have been shown to bind to and promote subsequent endocytosis of specific ligands such as intrinsic factor-cobalamin complex, albumin, immunoglobulin light chains, and high density lipoprotein, and transferrance (29). Our study is the first demonstration of BMP protein as a specific ligand for the CUB domain (Fig. 6). Further examination of whether the CUB-mediated BMP binding/antagonism could be extended for other members of the TGF-β superfamily will be interesting.

Our deletion analysis and biochemical / molecular data are broadly in line with genetic evidence (14), thus confirming that the 6-cysteine repeat motifs and CUB domain are essential for the SCUBE function in attenuating BMP activity when co-expressed in the same cells (Fig. 7). Furthermore, the permissive nature for the full-length SCUBE products may require as-yet-unidentified cofactors, possibly proteases, to proteolytically release the active C-terminal fragment for its anti-BMP activity when present in the same cells. The signaling activities of BMPs could be antagonized by a diverse group of
binding proteins, including Noggin and Chordin (30). The three-dimensional structure of the Noggin-BMP-7 complex revealed that Noggin binds as a dimer to a BMP dimer, occluding the interfaces of the binding domains for its receptors (31). Because of no sequence homology between the SCUBE1 C-terminal CUB domain and Noggin, a different mechanism may operate in the case of the SCUBE1-BMP2 complex. However, the precise mechanism by which SCUBE1 exerts its suppressive effects on BMP signaling remains to be further investigated.

Consistent with its in vitro anti-BMP activity, the Scube1 gene by targeted disruption in the mouse results in the brain patterning defects (Fig.s 8-10). Similar defects are seen in mice lacking the BMP antagonist Noggin with enhanced signaling through BMPs, central regulators of neural tube patterning (32,33). At E12.5, we found the SCUBE1 protein predominantly expressed in the cranial neural tube, with a lower level in the head mesenchyme, which implies its role in craniofacial development (Fig. 8C). Consistently, Scube1<sup>Δcub/Δcub</sup> embryos were characterized by abnormal hyperplasty in the cranial ganglia and a thick-walled neuroepithelium in the forebrain. Furthermore, the increase in neuroepithelial wall thickness coincided with excessive cell proliferation in the forebrain neuroepithelium, collapsing the third ventricle into a small canal-like structure (Fig.s 8C and 10F). Because complex and dynamic interactions between the neuroepithelium and the head mesenchyme are required for cranial neural tube morphogenesis, defects in one region of the developing neural tube may alter the neurolation process in other cranial regions, eventually affecting the entire cascade of craniofacial development (34). Thus, the forebrain defects may block the initiation of neural tube closure at the forebrain/midbrain boundary. Despite this morphogenetic failure, the midbrain neuroepithelium continues to proliferate in exencephalic Scube1<sup>Δcub/Δcub</sup> embryos and becomes a large convoluted mass of brain tissue protruding from the head (Fig. 10B).

Because exencephaly could result from defects in neural crest emigration, impaired closure of the cranial neural tube, altered neuroepithelial proliferation and/or programmed cell death of neurons or neuronal precursor cells (34), it is possible that hyperproliferation of neuroepithelium seen in the Scube1-mutant E12.5 embryos might be a secondary phenotype caused by above-mentioned developmental processes. Further analysis of earlier E8.5 to E10.5 embryos is needed to elucidate the primary defect in developing brain caused by the disruption of the Scube1 gene.

Human cranial neural tube closure defects (NTDs) are among the most common types of lethal birth defects, with a frequency of 0.2-3.5 per 1000 births depending on ethnicity and geographical location (35). Studies of mouse mutant models with various NTDs has provided some potential cellular and molecular mechanisms underlying these birth defects; however, no mutations identified in mouse have yet been shown to represent major genes for NTDs in humans. Thus, SCUBE1 may be a candidate for human NTDs, and the Scube1 mutant mice could provide a novel animal model
for studying the cellular, molecular and developmental mechanisms involved in the generation of NTDs.

In summary, the present molecular dissection of the SCUBE1 molecule has clearly defined the specific domains critical for membrane association, cell-cell adhesion, and BMP binding/antagonism, respectively. Further structural and functional studies of these domains will provide insights to better understand the molecular action and regulation of this novel multi-functional, multi-domain surface protein SCUBE1 during development or postembryonic biological processes. A conditional allele of the *Scube1* gene is important to further investigate the stage-specific role during the brain development and the tissue-specific function in adulthood.

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FOOTNOTES

*These authors contribute equally to this work.

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FIG. LEGENDS

**Fig. 1. Molecular analysis for the structure required for the secretion and cell-surface association.**

(A) Domain structure of SCUBE1 full-length (FL) and its deletion constructs (D1-D7). A Flag epitope tag was added immediately after the signal peptide sequence, thus at the N-terminus for easy detection. FL, amino acids 1-988; D1, amino acids 1-789; D2, amino acids 1-411; D3, amino acids 402-988; D4, amino acids 628-988; D5, amino acids 791-988; D6, amino acids 628-788; D7, amino acids 402-627. SP, signal peptide; E, EGF-like repeat; Cys-rich, cysteine-rich motifs; CUB, CUB domain. (B) The spacer region is essential for the secretion of the expressed SCUBE1 protein. HEK-293T cells were transfected with the indicated expression vector. Two days after transfection, samples from conditioned medium or cell lysates were immunoprecipitated by anti-Flag antibody and separated by SDS-PAGE and transferred to...
polyvinylidene difluoride membranes. Recombinant SCUBE1 proteins were detected by western blotting with anti-Flag antibody. (C) The spacer region is necessary for the surface expression of SCUBE1. Twenty four hours after transfection, a set of transfected cells as above was detached and stained with anti-Flag antibody to determine the cell surface expression. Experiments were repeated three times with similar results. IP, immunoprecipitation; WB, western blot analysis.

**Fig. 2. Generation of cell lines stably expressing the SCUBE1 EGF-like repeat deletion mutants.** (A) Domain composition of EGF-like repeat mutants. The EGF-like repeats 1-9, 4-9, 7-9, or only 7-9-specific deletion (E1-9, amino acids 33-412; E4-9, amino acids 157-412; E7-9, amino acids 281-412; ΔE7-9, same as E1-9 but lacking amino acids 282-397) were fused to the B7 transmembrane domain (B7TM) to target the chimeric protein on the plasma membrane. An HA epitope tag was added to monitor the expression of these recombinant proteins. (B) Western blot analysis of the EGF-like repeat deletion constructs. A2058 melanoma stable lines expressing E1-9, E4-9, or E4-9 deletion constructs underwent western blotting with the anti-HA antibody to verify the expression of these recombinant proteins. (C) Surface expression of the EGF-like repeat deletion mutant proteins. A2058 stable cell lines were analyzed by flow cytometry with the anti-HA or anti-SCUBE1 antibody to confirm the surface targeting of these mutant proteins.

**Fig. 3. The EGF-like repeats 7-9 are sufficient for homophilic reciprocal interactions of SCUBE1 in a Ca\(^{2+}\)-dependent manner.** (A) Degree of aggregation in suspension of A2058 parental and stable lines. A2058 parental (Control) or EGF-like repeat mutant stable lines (E1-9, E4-9, E7-9, or ΔE7-9) were detached and allowed to aggregate in suspension culture in the presence or absence of Ca\(^{2+}\) in aggregation medium for 1 h. Aggregates were defined as clusters of four or more cells. (B) Representative micrographs of aggregates formed by A2058 parental control or EGF-like repeat mutant stable lines. The experiments were performed three times in duplicate with similar results. *, p < 0.01 vs control cells. Original magnification: 100X.

**Fig. 4. Recombinant EGF-like repeat fragment inhibits the SCUBE1-mediated cell aggregation.** (A) Domain structure of SCUBE1 with recombinant fragment. Diagram of the SCUBE1 protein shows the location of the recombinant GST fusion protein: GST-E4-9 (amino acids 157-412) and GST-Spacer (amino acids 402-632). (B) Purification of recombinant SCUBE1 protein fragments. GST-SCUBE1 fusion proteins purified from the soluble fraction of bacterial lysates with glutathione-Sepharose beads were analyzed by SDS-PAGE and stained with Coomassie brilliant blue. (C) Specific blocking of SCUBE1-mediated cell aggregation by recombinant EGF-like repeats 4-9 fragments. The 1-h aggregation of A2058/SCUBE1-E7-9 cells were performed in the absence (-) or presence of GST alone, GST-E4-9, or GST-Spacer recombinant protein (10 µg/ml) and expressed as the degree of aggregation (%). aa, amino acid number.
Fig. 5. The EGF-like repeats 7-9 could mediate the oligomerization of SCUBE1. The Flag.SCUBE1-D2 mutant containing only the EGF-like repeats 1-9 (see Fig. 1) was expressed together with HA.SCUBE1-E1-9, -E4-9, or -E7-9, or -ΔE7-9 construct by transient transfection in HEK-293T cells. Detergent lysates were immunoprecipitated with the anti-HA antibody and then immunoblotted with the anti-Flag antibody to determine the associated proteins. Cell lysates were also immunoblotted to confirm the protein expression level for each construct. IP, immunoprecipitation; WB, western blot analysis.

Fig. 6. The C-terminal region of SCUBE1 could specifically interact with BMP2. (A) Domain organization of constructs used in this experiment. All constructs are as described in Fig. 1, except a new deletion construct, SCUBE1-ty97 mutant (amino acids 1-622), was made by mimicking a null mutant allele (ty97) in the zebrafish Scube2 (13-15). (B) Interaction between SCUBE1 and BMP2. The BMP2 expression construct (Myc-tagged) was transfected alone or in combination with the expression plasmids encoding indicated Flag-tagged SCUBE1 proteins in HEK-293T cells. Two days later, cell lysates underwent immunoprecipitation (IP) and western blotting (WB) with antibodies as indicated to determine the protein-protein interactions. ProBMP2, precursor BMP2.

Fig. 7. The C-terminal region of SCUBE1 acts as a BMP antagonist. (A) Design of a co-culture assay to assess the effect of SCUBE1 co-expression on the BMP2 signaling activity. Conditioned media from signaling cells (HEK-293T) co-transfected with BMP2 alone or together with various SCUBE1 expression plasmids as indicated were added to the responding cells (HepG2) that contained the BMP-responsive luciferase reporter plasmid I-BRE-Luc (16). (B) Co-expression of SCUBE1-D4 mutant attenuates the BMP2 signaling activity. BMP2 protein expressed alone or in combination with SCUBE1-FL, ty97, D5, or D6 in conditioned media of transiently transfected HEK-293T cells are active in this signaling assay. However, BMP2 activity can be attenuated by co-expression of the SCUBE1-D4 mutant. (C) SCUBE1-D4 mutant inhibits BMP2 precursor processing. HEK-293T cells were transfected with indicated expression plasmids for BMP2 and SCUBE1 mutants. Western blotting of the cell lysates and of the conditioned medium shows that transfected BMP2 precursor is cleaved into active, mature BMP2, which is markedly inhibited in the presence of the SCUBE1-D4 mutant, but not with all other mutant SCUBE1 forms. ProBMP2, precursor BMP2.

Fig. 8. Targeted disruption of the Scube1 gene. (A) The structure of the mutated Scube1 allele and the potential mutant transcript are shown. The insertional targeting vector obtained from a public resource (17) contains a 8-kb genomic fragment in the 3’Hprt (MHPP) vector carrying the puromycine resistance
(PURO), a minigene for the coat-color marker agouti (A), and exons 3-9 corresponding to split \textit{Hprt} minigene elements. After homologous integration of the \textit{ClaI}-digested targeting vector, the expected genomic structure of the targeted \textit{Scube1} locus is shown below as the “Mutated” locus. Exon duplication (exons 11-16) is created by the insertion of the vector. The boxes filled with black are endogenous exons of \textit{Scube1} and the open boxes are exons from the targeting vector. The 6-cysteine repeat motifs and the CUB domain are encoded by exons 16-19 and 20-21, respectively. The probe used for the Southern blot analysis is indicated by a filled bar and the sizes of the \textit{NarI} bands in the wild-type and mutated \textit{Scube1} alleles are also indicated. The vector backbone also carries a splice acceptor (SA) site that results in gene disruption by terminating transcription, missing the functional cysteine-rich repeat motifs and the CUB domain (bottom panel). The expression of the mutant transcript was confirmed by direct sequencing of the PCR product amplified by use of a primer pair (F2/R2) located specifically within the mutant transcript. (B) Southern blot analysis of genomic DNA from embryonic stem (ES) cell clones or genomic DNA from littersmates. Hybridization with the probe yielded a 24- or 5.4-kb band from the wild-type or mutant allele, respectively, as indicated. We also used PCR analysis of the ES cell or littersmate genomic DNA with one primer pair (F1/R1, see Panel A) designed from the vector backbone and the other one designed from exon 15 outside the targeting vector. Targeted ES clones and littersmates contain both primers providing a successful amplification of the 4.3-kb product. (C) Absence of the SCUBE1 immunoreactivity in neuroepithelium of E12.5 mutant embryos. Transverse sections from wild-type (+/+, left panel) and \textit{Scube1}^{\Delta \text{cub}/\Delta \text{cub}} mice (\Delta \text{cub}/\Delta \text{cub}, right panel) cut at the level of the eye were stained with anti-CUB domain-specific monoclonal antibody (6). SCUBE1 immunoreactivity is predominantly present in wild-type cranial neuroepithelial layer (brown) but absent in the \textit{Scube1}^{\Delta \text{cub}/\Delta \text{cub}} animals caused by the targeted deletion of C-terminal domain. h, hindbrain; f, forebrain; gV, trigeminal ganglion; gVIII, vestibulocochlear ganglion. Bars: 1 mm.

**Fig. 9. Acrania and defects of skull bones in the \textit{Scube1}^{\Delta \text{cub}/\Delta \text{cub}} mice.** (A) Lateral view of wild-type (+/+) and \textit{Scube1}-mutant (\Delta \text{cub}/\Delta \text{cub}) neonates. Note the absence of the cranium (between arrows) and the lack of brain tissue. (B, C) Skull defects in the \textit{Scube1}^{\Delta \text{cub}/\Delta \text{cub}} mice. Lateral view of wild-type (B) and \textit{Scube1}^{\Delta \text{cub}/\Delta \text{cub}} (C) skulls stained with alcian blue for cartilage and alizarin red for ossified bone. Note that cranial vault is missing in the mutant (asterisk). Only remnants of the frontal, parietal, squamosal and alisphenoid bones remain in the mutant. The interparietal and supraoccipital bones are absent. Asterisk denotes exencephaly in the midbrain region.

**Fig. 10. Exencephaly in \textit{Scube1}-mutant mice as a result of an early neural-tube closure defect.** (A, B) Lateral view of E12.5 wild-type (A) and \textit{Scube1}^{\Delta \text{cub}/\Delta \text{cub}} (B) embryos. The mutant shows an overproliferation of neural tissue and the everted cranial neural folds (between arrows) in the midbrain region. (C, D) Transversal sections stained with hematoxylin and eosin through the midbrain at E12.5,
depicting the everted neuroepithelium (asterisks) and neural tube closure defects in the Scube1Δcub/Δcub animal. (D) compared with wild-type control (C). m, midbrain. (E, F) Transverse forebrain sections of the Ki-67-stained wild-type (E) and Scube1Δcub/Δcub (F) E12.5 embryos. Brown nuclei are Ki-67-positive cells and indicate active cell proliferation. Sections were counterstained with hematoxylin to detect all nuclei. Bars: 500 µm.
FIG. 5

| Vector          | + |   |   |
|-----------------|---|---|---|
| HA.SCUBE1-E1-9  | + |   |   |
| HA.SCUBE1-E4-9  | + |   |   |
| HA.SCUBE1-E7-9  | + |   |   |
| HA.SCUBE1-ΔE7-9 | + |   |   |
| Flag.SCUBE1-D2  | + | + | + |

Flag.SCUBE1-D2

IP: HA; WB: Flag

HA.SCUBE1-E1-9
HA.SCUBE1-E4-9
HA.SCUBE1-E7-9
Flag.SCUBE1-D2

IP: HA; WB: HA
IP: Flag; WB: Flag
FIG. 6

A

B

| Vector    | + | + | + | + | + | + |
|-----------|---|---|---|---|---|---|
| SCUBE1-FL | + |   |   |   |   |   |
| SCUBE1-ty97| + |   |   |   |   |   |
| SCUBE1-D4 | + |   |   |   |   |   |
| SCUBE1-D5 | + |   |   |   |   |   |
| SCUBE1-D6 |   |   |   |   |   |   |
| BMP2      |   |   |   |   |   |   |

IP: BMP2; WB: SCUBE1

WB: BMP2

WB: SCUBE1
FIG. 7

A

(1) Transfection
Signaling cells
(HEK-293T)

SCUBE1
+
BMP2

Conditioned media

Responding cells
(HepG2)

Luciferase reporter

(2) Co-culture

(3) Luciferase activity assay

B

Vector
BMP2
SCUBE1-FL+BMP2
SCUBE1-ty97+BMP2
SCUBE1-D4+BMP2
SCUBE1-D5+BMP2
SCUBE1-D6+BMP2

Relative luciferase activity

0.0  0.5  1.0  1.5  2.0  2.5

C

| Vector  | + | Vector  | + | Vector  | + | Vector  | + |
|---------|---|---------|---|---------|---|---------|---|
| SCUBE1-FL | + | SCUBE1-ty97 | + | SCUBE1-D4 | + | SCUBE1-D5 | + | SCUBE1-D6 | + |
| BMP2 | + | + | + | + | + |

mature BMP2
(22 kDa)

Conditioned media
(WB: BMP2)

proBMP2
(~60 kDa)

Lysates
(WB: BMP2)

200
130
79
39
31

Lysates
(WB: SCUBE2)
Domain and functional analysis of a novel platelet-endothelial cell surface protein, SCUBE1
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