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Molecular characterization of GDD1/TMEM16E, the gene product responsible for autosomal dominant *gnathodiaphyseal dysplasia*

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Abstract

The human *GDD1/TMEM16E* gene has been found to be mutated in *gnathodiaphyseal dysplasia*, an unusual skeletal syndrome with autosomal dominant inheritance. The molecular and biochemical function(s) of GDD1 protein has not yet been elucidated. In this study, we examined the murine *GDD1* gene expression pattern during embryonic development, and characterized the cellular and tissue localizations of its gene product using a GDD1-specific antibody. In the developing embryos, *GDD1* mRNA expression was principally associated with differentiating and developing somites, with a highly complex spatiotemporal pattern that involved the myotomal and sclerotomal lineages of somites. Biochemical studies indicated that GDD1 protein is an integral membrane glycoprotein that resides predominantly in intracellular vesicles. Immunohistochemical analysis showed a high level of murine GDD1 protein expression in cardiac and skeletal muscle tissues, and in growth-plate chondrocytes and osteoblasts in bone. These observations suggest diverse cellular role(s) of GDD1 in the development of musculoskeletal system.

Keywords: *Gnathodiaphyseal dysplasia*; GDD1; TMEM16

Gnathodiaphyseal dysplasia (GDD; MIM 166260) is an unusual generalized skeletal syndrome inherited in an autosomal dominant fashion [1,2]. Typically, patients with this rare syndrome show increased bone fragility, sclerosis/bowing of tubular bones, and fibro-osseous lesions of the jawbones with a prominent psammomatoid body component. Patients also have a propensity for jaw infection and often suffer from persistent and recurrent purulent osteomyelitis.

Recently, using a positional cloning approach, we have discovered a novel *GDD1* gene that is mutated in the original Japanese GDD family and also in an African–American family [2]. The human *GDD1* gene maps to chromosome 11p14.3–15.1, and encodes a 913 amino-acid protein whose molecular functions and biochemical properties have not yet been characterized. Interestingly, *in silico* analysis revealed that GDD1 exhibits a significant similarity to the newly identified TMEM16 protein family [3]. The TMEM16 proteins constitute a large family found in all eukaryotes, and, in mammals, are now known to be composed of at least 10 members including GDD1/TMEM16E. They share a common predicted eight-transmembrane (TM) topology with N- and C-terminal tails facing the cytoplasm, and a conserved domain referred to as the DUF590 (Domain of Unknown Function 590, PFAM Accession No.: PB175613). To date, none of the vertebrate TMEM16 proteins have been functionally characterized,

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and there are not enough research data available, except for several gene expression profiles reported by our group and others [4–6].

To our knowledge, among members of the *TMEM16* family, *GDD1* is the first gene for which a disease-related mutation has been identified. As an important step toward elucidating the role of *GDD1* in the pathogenesis of GDD, and to provide clues to understanding the molecular functions of other TMEM16 members, we conducted a series of experiments aimed at detecting the *GDD1* gene expression pattern during murine embryonic development, and characterizing the cellular and tissue localizations of its gene product.

Materials and methods (see Supplementary information for further details)

Cell cultures and animals. The myoblast cell-lines (L6, Sol8, and C2C12) were cultured under standard conditions [4]. To induce myogenic differentiation, subconfluent cultures were switched to low-serum differentiation medium consisting of DMEM and 2% horse serum. BDF1 (*Jcl:BDF1*), the Dystrophin-deficient MDX (*C57BL/10-mdx*), and their age-matched *C57BL/10* mice were obtained from CLEA Japan Inc. All animal experiments were conducted under protocols approved by the IACUC.

In situ hybridization (ISH). The murine cDNA fragments for *GDD1*, *Myogenin*, and *PAX1* were amplified from an E11.5 embryo. To generate anti-sense or sense cRNA probes, a T7-promoter sequence was attached to the 5′-ends of the lower or upper primer, respectively. RT-PCR products were directly used for the *in vitro* transcription reaction with T7 RNA polymerase (Toyobo) in the presence of DIG-labeled UTP (Roche). Embryos at different developmental stages were obtained by dissection of pregnant BDF1 mice. Whole-mount ISH was performed according to standard methods. Section ISH was carried out with an automated Ventana Discovery Instrument (Ventana). For *GDD1* mRNA, the signal was amplified using the AmpMap Kit with TSA.

Generation of the anti-GDD1 antibodies. The N- and C-terminal segments of mouse GDD1, residues 80–281 (GDD1N) and 854–899 (GDD1C), were subcloned to either the pMAL-c2X MBP (NEB) or the pGEX-6P GST (Amersham) vectors. MBP fusion proteins, MBP-GDD1N and MBP-GDD1C, were prepared by transforming BL21DE3 (Invitrogen) with the relevant pMAL constructs. After induction of recombinant protein expression with IPTG, cells were lysed and centrifuged to separate the soluble and insoluble fractions. Since MBP-GDD1N fusion protein was expressed only in the insoluble fraction, insoluble materials were solubilized in 8 M urea-containing buffer and, thereafter, the urea was removed by dialysis. The MBP fusion proteins were purified by affinity-chromatography using an amylose resin (NEB). Preparation of the GST fusion proteins was carried out using Glutathione Sepharose-4B (Amersham). The purified MBP fusion proteins were used to immunize rabbits to produce polyclonal antisera. The anti-MBP-GDD1N antibodies were affinity-purified by passing the serum over a column consisting of the immunizing antigen conjugated to CNBr-activated Sepharose-4B beads (Amersham), followed by passage through a MBP/Sepharose-4B column to absorb anti-MBP antibodies. The anti-MBP-GDD1C antibodies were purified by affinity-chromatography on a GST-GDD1C/Sepharose-4B column.

Subcellular and membrane fractionation. Subcellular fractionation experiments were performed in three different ways. (A) *Differential centrifugation.* Differentiated L6 myotubes were homogenized in hypotonic Hepes buffer and centrifuged at 1000*g* for 10 min. The pellet was washed in hypotonic buffer containing 1% Triton X-100 to purify P1 nuclear fraction. The postnuclear S1 supernatant was subjected to centrifugation at 7000*g* for 10 min to generate a P2 (mitochondria/microsomal) pellet. The resulting S2 supernatant was centrifuged at 100,000*g* for 1 h yielding the P3H pellet enriched with high-density microsomes (HDM). The remaining S3 supernatant was finally centrifuged at 166,000*g* for 2 h to produce the low-density microsome (LDM)-enriched P3L pellet and the cytosolic (S) fraction. (B) *Iodixanol gradient fractionation.* L6 myotubes were homogenized in STE buffer and centrifuged at 7000*g* for 10 min. The supernatant was centrifuged at 166,000*g* for 2 h to produce the microsome-enriched pellet. The pellet was adjusted to 50% (w/v) iodixanol (OptiPrep), bottom loaded on a 10–30% gradient, and then centrifuged at 100,000*g* for 2 h. Following centrifugation, fractions of 500 μl were collected from the bottom. (C) *Membrane isolation* [7]. Tissues or cells were homogenized in HES buffer and centrifuged at 19,000*g* for 20 min. The resultant pellet was resuspended, layered on top of a 1.12 M sucrose cushion, and centrifuged at 100,000*g* for 1 h. The interphase (containing plasma membrane [PM]) was collected and repelleted. The supernatant from a 19,000*g* centrifugation was centrifuged at 41,000*g* for 20 min to obtain a HDM membrane. The 41,000*g* supernatant was finally centrifuged at 180,000*g* for 1.5 h to obtain the LDM membrane.

Western blotting. Protein extracts were electrophoresed in SDS–PAGE gels, transferred to a PVDF membrane, and blocked with 10% skim-milk. Membranes were incubated with primary antibodies overnight at 4 °C. Immunoreactive bands were visualized using the ECL-Plus system (Amersham). To determine the nature of membrane associations involving GDD1, the microsomal extracts were treated with either 1% SDS, 1% NP-40, 1% Triton X-100, 0.2 M Na_2CO_3 pH 11.0, or 1.5 M NaCl, and repelleted by centrifugation. To assess the glycosylation status of GDD1, membrane fractions were treated with either PNGase-F (NEB) or Endo-H (NEB), and subjected to immunoblotting to detect changes in the electrophoretic mobility of the GDD1 protein band.

Immunohistochemistry. Tissue samples of cardiac and skeletal muscles were processed into paraffin-wax, and 5-μm thick sections were prepared. Additional mouse sections of adult femoral head cartilage and embryonic long bones (E18.5) were obtained from commercial sources. Immunohistochemical staining was performed using HISTOMOUSE-PLUS kits (Zymed). Primary antibodies were used at the following dilutions: anti-GDD1 pAbC at 1:100; anti-GLUT4 at 1:500; anti-Dystrophin at 1:20; anti-Type-X collagen at 1:200; and anti-Type-II collagen at 1:200.

Results

GDD1 expression during murine embryogenesis

By whole-mount ISH, we found that *GDD1* expression became detectable at E9.5 of murine development and, later, it increased progressively in developing somites (Fig. 1A shows representative images from an E11.5 embryo). On a transverse section of an E9.5 embryo, *GDD1* expression was confirmed in myotomal (*Myogenin*-positive), but not in sclerotomal (*PAX1*-positive) or dermomyotomal, cells (Fig. 1B). This myotomal expression of *GDD1* persisted through E11.5; however, it was no longer seen at E12.5, while *Myogenin* continued to be expressed. The expression pattern thereafter indicated that myotomal *GDD1* expression continued in myotome-derived muscle progenitor cells migrating to limb buds (Fig. 1D; asterisks). On the contrary, in a transverse section at E12.5, a weak but significant level of *GDD1* expression was detected in the perichondrium of the developing vertebral body, a part of the sclerotome derivative positive for *PAX1*, and it was even more evident at E13.5 (Fig. 1B). On the longitudinal section, *GDD1* expression was seen in the posterior ventromedial compartment of the future vertebral bodies and intervertebral discs along the entire rostro-caudal axis,

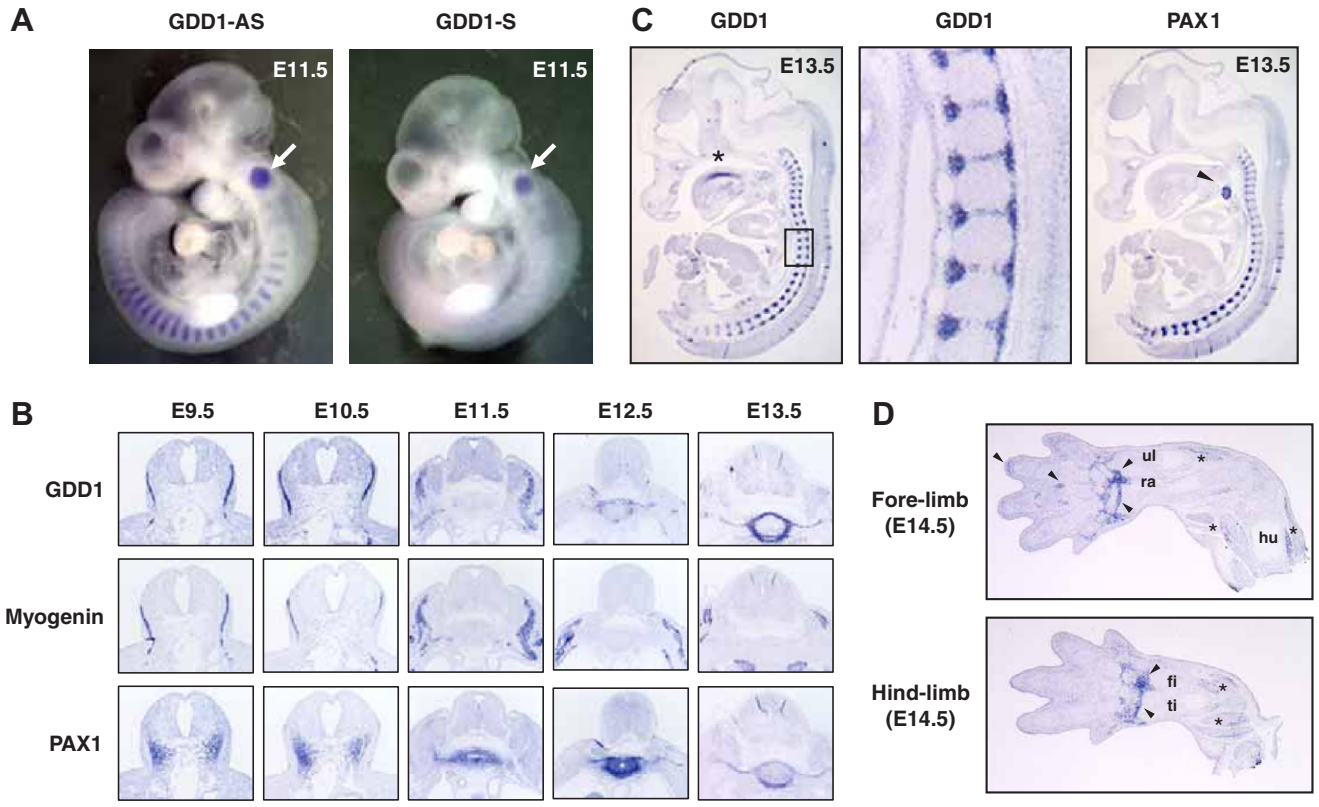

Fig. 1. Expression of *GDD1* mRNA during embryogenesis. (A) Lateral views of whole-mount ISH of E11.5 embryos with anti-sense (AS) and sense (S) *GDD1* probes. Segmental bands indicative of *GDD1* expression are seen. Arrows indicate nonspecific signals in the otic vesicles. (B) Transverse sections of E9.5–E13.5 embryos probed for *GDD1*, *Myogenin*, and *PAX1*. (C) Midsagittal sections of E13.5 embryos probed for *GDD1* and *PAX1*. The middle panel is a higher magnification of the skeleton in the left panel, showing *GDD1* expression in the intervertebral mesenchyme and the perichondrium of vertebral bodies. *GDD1* transcripts are also detected in the developing tongue (asterisk), while *PAX1* is detected in the thymus (triangle). (D) Sections of an E14.5 embryo showing *GDD1* expressions in the fore- and hind-limbs. Positive signals are present in muscle (asterisks) and in the mesenchyme surrounding the digits and carpal/tarsal bones (triangles).

reaching its maximal level at E13.5 (Fig. 1C). *PAX1* showed a similar pattern of expression, although its peak expression preceded that of *GDD1* by approximately 24 h (Fig. 1B). In other tissues, there were notable expression differences between the two genes (e.g., *PAX1* expressions in the thymus; Fig. 1C). At E14.5 and later stages, *GDD1* expression in ventromedial sclerotomal cells was gradually decreased and was almost abolished by E16.5 (data not shown). In E14.5 limbs, *GDD1* expression was also demonstrated in the mesenchymal tissues surrounding the cartilaginous anlages of digits and carpal/tarsal bones (Fig. 1D; triangles), while only background expression levels were seen in developing limb-bones.

Characterization of anti-GDD1 antibodies

A preliminary topological model of GDD1 was previously proposed (Supplementary Figure S1A; [2]). To generate polyclonal antibody specific for GDD1, recombinant MBP fusion proteins of either the N- or C-terminus of mouse GDD1 (GDD1N and GDD1C) were prepared and used to immunize rabbits. Two antibodies, named anti-GDD1 pAbN and pAbC (directed against GDD1N and GDD1C, respectively), were obtained, and their specificities were tested by immunoblotting. Both antibodies readily reacted with their corresponding immunizing antigens fused to GST (Figure S1B), indicating that these recognize the GDD1N and GDD1C segments as epitopes. We have previously observed that, in C2C12 cells, *GDD1* mRNA is low or absent under normal culture conditions but is robustly induced during myogenesis [4]. We therefore prepared protein extracts from differentiated myotubes (C2C12, Sol8, and L6) and analyzed them by immunoblotting. Both pAbN and pAbC antibodies detected a prominent immunoreactive band at ~100 kDa in extracts from differentiated but not undifferentiated cells, a result in good agreement with the calculated molecular mass of GDD1 protein (a representative immunoblot obtained with pAbC antibody is shown in Fig. 2A). The protein band was specifically blocked by preincubation of the antibodies with corresponding MBP fusion proteins (data not shown). Further, similar results were obtained using extracts from mouse cardiac and skeletal muscle tissues (Fig. 2B). From these observations, we concluded that the anti-GDD1 pAbN and pAbC antibodies provide sufficient sensitivity and specific-

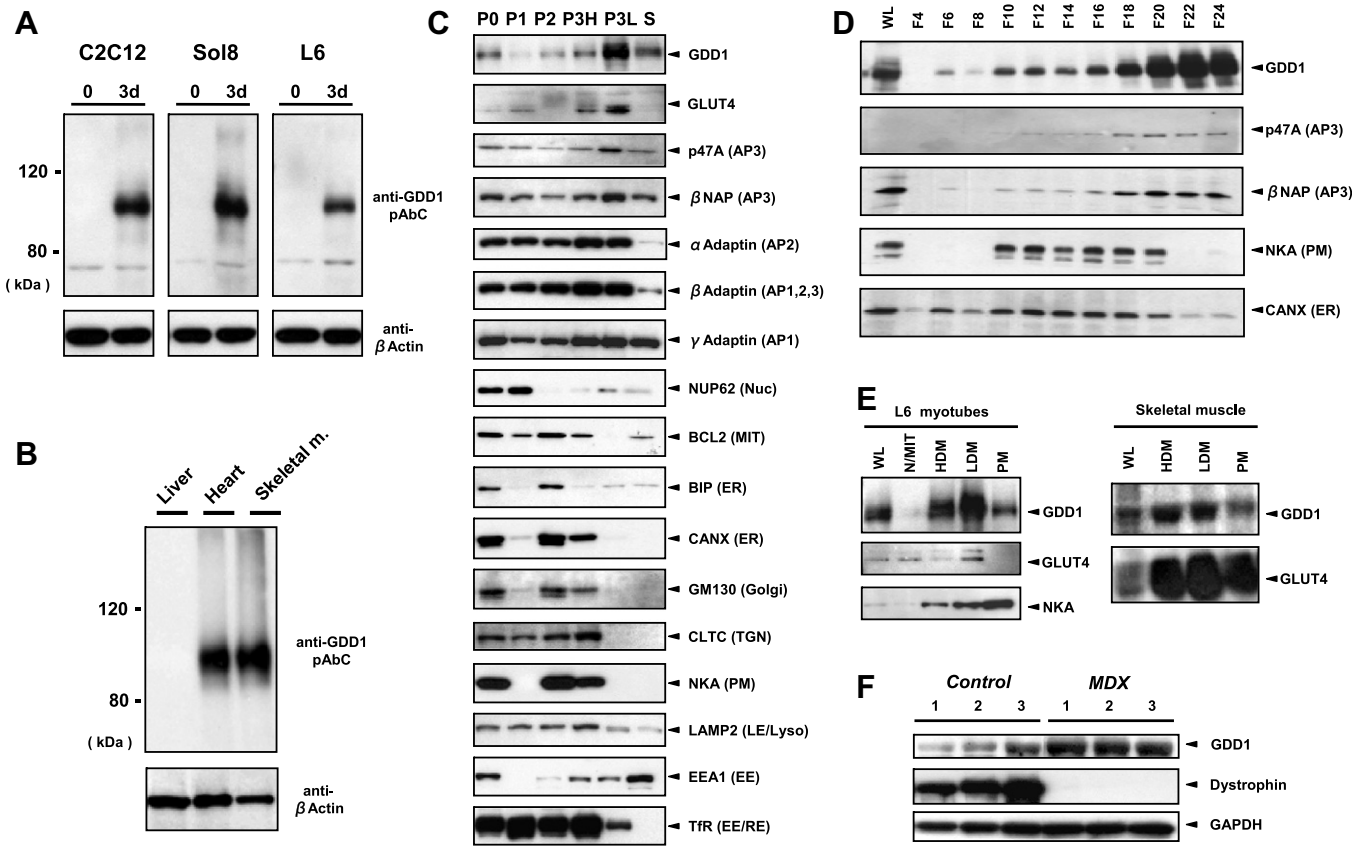

Fig. 2. Biochemical properties of GDD1 protein. (A) Induction of GDD1 protein expression during myogenesis (C2C12, Sol8, L6). Whole-cell extracts prepared from undifferentiated (0; Day 0) and differentiated (3d; Day 3) cells were subjected to immunoblotting with the anti-GDD1 pAbC. The same blots were re-probed with anti-βActin antibody to confirm equal protein loading. (B) GDD1 protein expression in tissues. Tissue extracts from liver, heart, and skeletal muscle were subjected to immunoblotting using either anti-GDD1 pAbC or anti-βActin antibody. (C) Subcellular fractionation by differential centrifugation. Total cell homogenates (P0) prepared from differentiated L6 myotubes were subjected to sequential differential centrifugation generating the P1 (nuclear), P2 (mitochondria/microsomal), P3H (HDM), P3L (LDM), and S (cytosolic) fractions. Proteins of each fraction were assessed by immunoblotting using antibodies against GDD1 (pAbC; top) and various marker proteins of organelles (indicated in parentheses; lower 16 panels). GLUT4, Glucose transporter-4; p47A, Clathrin-associated adaptor protein homolog; βNAP, Adaptin β3B; NUP62, Nucleoporin-62; BCL2, B-cell leukemia/lymphoma-2; BIP, Binding protein/grp78; CANX, Calnexin; GM130, Golgi Matrix Protein-130; CLTC, Clathrin; NKA, Na/K ATPase; LAMP2, Lysosomal-associated membrane protein-2; EEA1, Early endosome antigen-1; TfR, Transferrin receptor-1; AP3, adaptor protein complex-3; AP2, adaptor protein complex-2; AP1, adaptor protein complex-1; Nuc, nucleus; Mit, mitochondria; ER, endoplasmic reticulum; Golgi, Golgi apparatus; PM, plasma membrane; LE/Lyso, late endosome/lysosome; EE, early late endosome; RE, recycling endosome. (D) Fractionation in iodixanol density gradients. A microsome-enriched fraction from L6 myotubes was separated on a continuous 10–30% iodixanol gradient. Fractions (F1–25) were collected, starting from the bottom of the gradient, and subjected to immunoblotting. Only even-numbered fractions between F4 and F24 are shown here. (E) Membrane isolation. WL, whole tissue or cell extracts; N/MIT, nuclear/mitochondrial membrane; HDM, high-density microsomal membrane; LDM, low-density microsomal membrane; PM, plasma membrane. Equal amounts of protein from each fraction were assessed by immunoblotting. (F) GDD1 expression in dystrophic skeletal muscles. Muscle extracts were prepared from three control and three dystrophic MDX mice, and subjected to immunoblotting with antibodies to GDD1, Dystrophin and GAPDH. No Dystrophin protein is detectable in the skeletal muscles from MDX mice, whereas increased expressions of GDD1 are apparent.

ity to detect endogenous GDD1. We selected the pAbC for the following studies because it preferentially recognized the GDD1 protein at lower concentrations.

Biochemical characterization of GDD1

Our previous studies of COS-7 cells transiently transfected with a carboxy-terminal V5-epitope-tagged human GDD1 expression vector demonstrated the endoplasmic reticulum (ER) localization of GDD1 [2]. To examine the subcellular localization of endogenous GDD1 in greater detail, we performed several types of biochemical fractionation experiments. First, homogenates prepared from differentiated L6 myotubes were subjected to differential centrifugation to prepare crude subcellular particles (Fig. 2C; P0, total cell lysates; P1, nuclei; P2, mitochondria/microsome; P3H, HDM; P3L, LDM; S, cytosol), and each particle was assayed by immunoblotting for the presence of specific organelle marker proteins to provide indications of the organelle composition. Under our experimental conditions, the GDD1 distributed within the P2 to S fractions and was significantly enriched in the P3L fraction (Fig. 2C). GDD1 was also detected in the cytosolic fraction; however, since the highest probability was that

of GDD1 being an integral membrane protein, this was most likely due to cross-contamination of the other membrane-containing fractions. The distribution pattern of GDD1 was similar to those detected for GLUT4 and several subunits of the adaptor protein (AP) complex, especially p47A and βNAP. When pooled microsomal fractions were separated by iodixanol-based density gradient ultracentrifugation, again, the distribution of GDD1-containing subfractions was similar to those for AP proteins (Fig. 2D), while there were only partial overlaps with PM or ER proteins. Finally, to determine in which cellular membrane compartments GDD1 resides, membrane fractions from differentiated L6 myotubes and muscle tissues were prepared according to the method described by Mitsumoto et al. [7], which has been repeatedly shown to effectively separate different membrane fractions. Immunoblotting using the pAbC revealed GDD1 to reside predominantly in the LDM (containing Golgi apparatus, secretory vesicles, and endosomes) and HDM (containing ER, *trans*-Golgi network, and endosomes) membrane fractions, with smaller amounts in the PM fraction (Fig. 2E).

An integral association of GDD1 with the membrane was suggested by solubility experiments, in which GDD1 remained sedimentable after treatments with alkali and a high concentration of salts but was solubilized by both ionic and non-ionic detergents (Figure S2A). Furthermore, according to the difference in electrophoretic migration during SDS–PAGE, the GDD1 protein was shown to be PNGase-F sensitive but Endo-H insensitive, which indicated GDD1 to be a mature glycoprotein that had reached at least the *medial*- or *trans*-Golgi apparatus (Figure S2B).

We examined the protein expression levels of endogenous GDD1 in skeletal muscle obtained from Dystrophin-deficient MDX mice, which are characterized by progressive skeletal muscle weakness and abnormal degeneration–regeneration cycles of myofibers [8]. As shown in Fig. 2F, GDD1 protein levels were significantly elevated in dystrophic muscles.

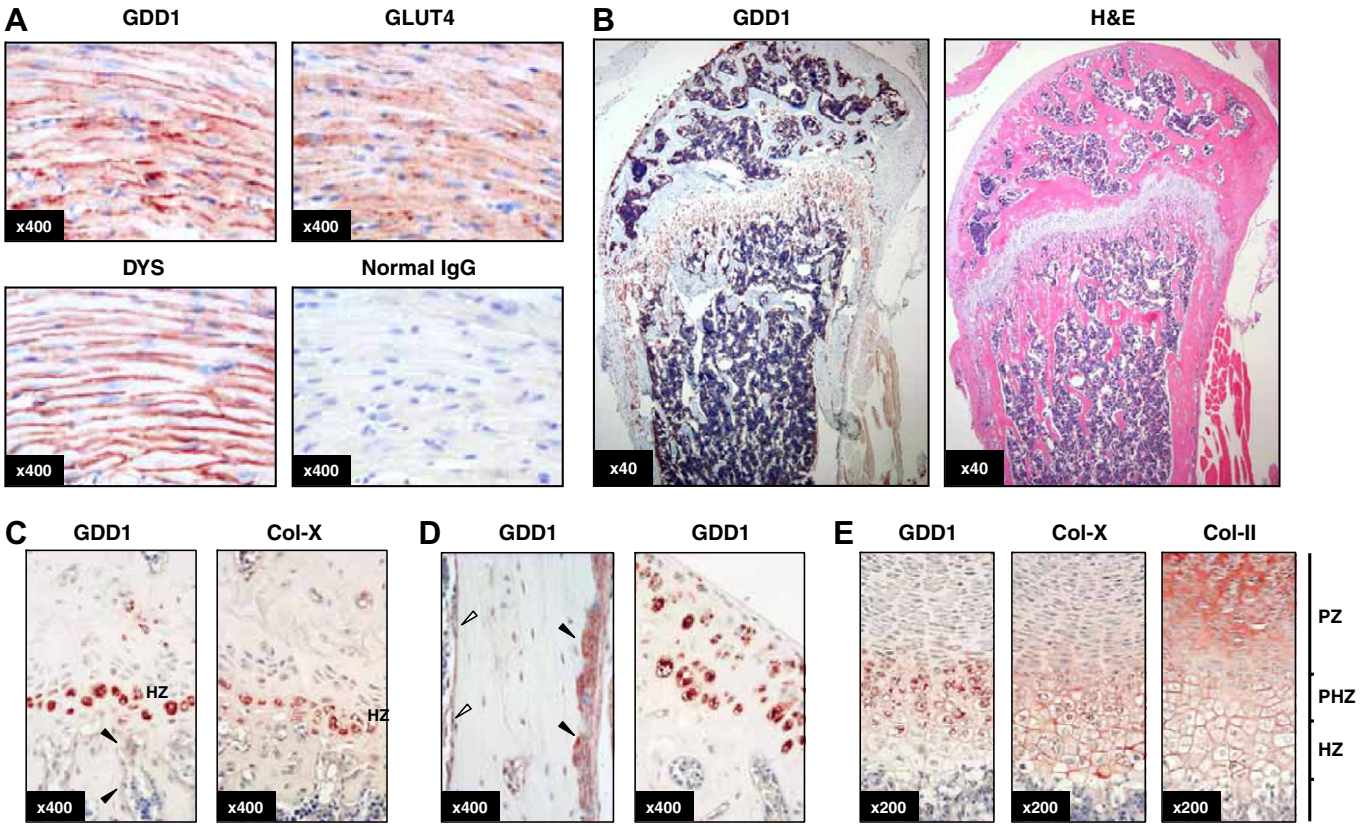

Fig. 3. Immunohistochemical localizations of GDD1. (A) Immunohistochemistry of GDD1 in adult mouse heart. Adjacent sections were subjected to immunohistochemistry using antibodies against GDD1 (pAbC), GLUT4, and Dystrophin. GDD1-immunoreactivity was visualized using the AEC chromogen substrate (red-reaction product), followed by counterstaining with hematoxylin. Staining with normal rabbit IgG served as a negative control. (B) Immunohistochemical staining of GDD1 in adult mouse femoral head cartilage. Histological appearances with hematoxylin–eosin (H&E) staining of the epiphysis and diaphysis are also shown. (C) GDD1-immunoreactivities in hypertrophic chondrocytes (left panel). Osteoblasts lining the trabecular bones are also positive for GDD1 (triangles). The right panel shows the distribution pattern of Type-X collagen (Col-X; a marker of chondrocyte hypertrophy). HZ, hypertrophic zone. (D) GDD1-immunoreactivities in osteoblasts lining the bone surfaces (right panel) and in articular cartilage cells (left panel). Positive GDD1 signals are detectable in both endosteal (open triangles) and periosteal osteoblasts (triangles). (E) The distal growth-plate of the tibia of an E18.5 embryo was immunostained for GDD1, Type-X collagen (Col-X) and Type-II collagen (Col-II; a marker of immature, proliferating chondrocytes). PZ, proliferating zone; PHZ, prehypertrophic zone; HZ, hypertrophic zone.

Immunohistochemical localization of GDD1

Immunocytochemistry of differentiating C2C12 cells with anti-GDD1 pAbC revealed the GDD1-immunoreactivity to be diffusely distributed over most of the fully differentiated myotubes with elongated, bipolar, and multinucleated cell appearing, but not in mononucleated undifferentiated myoblasts (Figure S3). We next examined the immunohistochemical localizations of GDD1 in adult mouse cardiac muscle and bone tissues, where the highest levels of *GDD1* mRNA were previously demonstrated [2]. In the heart, GDD1-immunoreactivity was confined to myofibers, and appeared to be associated with both cytoplasmic and subsarcolemmal vesicle-like structures (Fig. 3A). In this respect, on serial sections, the GLUT4-staining displayed a finer granular intracellular pattern as compared to that of GDD1, while strong staining of the sarcolemma was apparent for Dystrophin.

Immunohistochemistry of adult mouse femoral head cartilage revealed GDD1 to be expressed exclusively in hypertrophic chondrocytes of the epiphyseal growth-plate, in a pattern similar to that of Type-X collagen (Fig. 3B and C). Expression of GDD1 was also evident in osteoblasts lining mineralized bone surfaces, as well as in articular cartilage cells (Fig. 3C and D). Osteoclast-like multinucleated cells showed weak diffuse cytoplasmic staining, while osteocytes were negative. Other bone-marrow cells were essentially negative for GDD1, except for some types of leukocytes (e.g. eosinophils) showing nonspecific background signals. As no *GDD1* mRNA expression was confirmed by ISH in developing limb-bones (Fig. 1D), we performed an additional immunohistochemical study on embryonic bone sections, and confirmed weak but significant GDD1 protein expression in limb-bones of E14.5 embryos (data not shown). The reasons for the difference between mRNA and protein expression levels remain unclear; however, we assume that it might in part be due to the differences in turnover rates of *GDD1* transcripts and their corresponding proteins in these tissues. The GDD1 protein expression was more evident in growth-plates of the proximal/distal tibia and distal femur prepared from E18.5 embryos (Fig. 3E and Figure S4 show representative images of the distal tibial growth-plate). It is noteworthy that, at this embryonic stage, GDD1 protein expression was found to essentially be restricted to prehypertrophic zone chondrocytes rather than the terminal hypertrophic zone chondrocytes.

Discussion

The results of the ISH study showed *GDD1* mRNA to be expressed in a complex and dynamic pattern during murine embryogenesis. The *GDD1* expressions in the somite sclerotome and its derivatives appear to correlate directly with disease phenotype and suggest a potential role of GDD1 in bone-forming cells. Immunohistochemical analysis of adult mouse femoral head cartilage revealed GDD1 to be highly expressed in growth-plate chondrocytes, where endochondral ossification normally occurs, and in osteoblasts at sites of active bone turnover. Based on these findings, we speculate that altered bone-formation resulting from *GDD1* mutation(s) may contribute to the pathogenesis of GDD.

During embryogenesis, *GDD1* was also expressed exclusively in the myotomal component of somites and developing skeletal muscle cells. These observations were unexpected but in hindsight fit with our previous data showing higher levels of *GDD1* transcripts in heart and skeletal muscle tissues [2], and mRNA up-regulation during C2C12 myogenesis [4], as well as findings in the current study, in which we measured GDD1 protein levels in muscle tissues by immunoblotting. The possible role of GDD1 in muscle phenotype is further supported by immunoblotting data demonstrating a significantly increased level of GDD1 protein expression in the skeletal muscles of dystrophic MDX mice. In addition, a recent study showed that human skeletal *GDD1*/*TMEM16E* mRNA was differentially regulated after aerobic exercise training [9]. These findings suggest that GDD1 may play a role in the stimulation of both myogenic precursor cell proliferation and muscle hypertrophy/regeneration processes. Although alterations in skeletal muscle phenotype (e.g. muscle wasting and myopathy) have not been established in GDD patients, the possible role(s) of GDD1 in skeletal muscle activity might merit further investigation.

The results of biochemical analyses indicate that GDD1 protein is an integral membrane glycoprotein, residing predominantly within intracellular membrane vesicles, and also in the plasma membrane. These localization patterns of GDD1 were, at least partially, consistent with GDD1 localization patterns obtained in immunocytochemical and immunohistochemical studies. However, in this study, we were unable to definitively determine the precise nature of these intracellular GDD1-containing compartments, and further investigations are needed. It should be mentioned that these results may disagree with our previous assignment of GDD1 protein to the ER [2], which was based on experimental results obtained using COS-7 cells transfected with a human GDD1 construct. We speculate the ER localization of GDD1 might be the result of incorrect localization or artifacts that resulted from short-term overexpression or an artificial epitope-tag.

This study represents a first step towards the functional characterization of GDD1 protein, and further studies are clearly necessary to elucidate its molecular function. The resultant findings would aid in our understanding of the molecular mechanisms of GDD pathogenesis, as well as potential role(s) of other TMEM16 proteins in various human disorders.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc. 2007. 03.108.

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