RoBo-1, a Novel Member of the Urokinase Plasminogen Activator Receptor/CD59/Ly-6/Snake Toxin Family Selectively Expressed in Rat Bone and Growth Plate Cartilage*

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Using differential display polymerase chain reaction, we cloned a novel cDNA named RoBo-1 from rat tibia. RoBo-1 is abundantly expressed in bone, including the hypertrophic chondrocytes of the growth plate where cartilage is remodeled into bone. RoBo-1 mRNA expression increased in response to two modulators of bone metabolism, estradiol and intermittent mechanical loading, suggesting a role in bone homeostasis. The 1.6-kilobase cDNA encodes a 240-amino acid protein with a cysteine spacing pattern, suggesting that RoBo-1 is a novel member of the urokinase plasminogen activator receptor/CD59/Ly-6/snake toxin family. Furthermore, the C-terminal contains a glycosyl-phosphatidylinositol attachment site, suggesting that it is a cell surface protein similar to other mammalian members of this family. The strongest homology of RoBo-1 is to the snake serum-derived phospholipase A2 inhibitors, which uniquely contain two of the cysteine domains but are secreted proteins. Interestingly, RoBo-1 is likely the first membrane-anchored member of this family containing two cysteine domains. Thus, the tissue specificity, responsiveness to bone protective mediators, along with its relationship to the multifunctional urokinase plasminogen activator receptor/CD59/Ly-6/snake toxin family suggests that RoBo-1 may play a novel role in the growth or remodeling of bone.

To reduce the risk of fracture, the skeleton responds to local mechanical forces by forming new bone at these highly stressed sites (1). Maintenance of bone tissue is a unique process that probably requires specialized proteins. Identification of the bone-specific proteins involved in this process could enhance our understanding and ability to treat bone diseases such as osteoporosis. Despite years of intensive study, very few proteins have been identified that are expressed primarily in bone tissue. The development of molecular biology techniques based on differential expression of mRNA provides new opportunities to identify novel bone-specific proteins such as the recently described osteoclast protease cathepsin K (2). Using differential display polymerase chain reaction (DD-PCR) (3), we sought to identify new bone-specific proteins directly from whole bone tissue. We report here the discovery of a novel cDNA named RoBo-1 (Rodent Bone) that is expressed abundantly in bone, including the lengthening growth plate where cartilage is remodeled into bone, but is not significantly expressed in any other tissues examined.

Bone strength is regulated by a coupled process of formation and resorption. These processes are regulated by both systemic hormones and local factors. For instance, after menopause or ovariectomy there is rapid loss of bone due to a relative increase of resorption relative to bone formation (4). Administration of estrogen can prevent this loss of bone by restoring the balance of formation and resorption. However, mechanical forces are the predominant signal that determine the local architecture needed to provide optimum strength (5). The critical need for mechanical load is most obviously seen during immobilization and space flight where bone is lost rapidly despite normal hormone levels (6). Conversely, the application of repetitive forces to bone results in additional bone tissue being added to the mechanically stressed site to maintain the structural integrity of the bone (7, 8). Interestingly, we have observed that RoBo-1 mRNA levels increased after both estrogen treatment and with repetitive mechanical loading regimes in rats.

RoBo-1 is a new member of the emerging multifunctional uPAR/CD59/Ly-6/snake toxin family (9). Despite a diverse array of functions, the family is characterized by a motif of 8–10 similarly spaced cysteines forming a common three-dimensional structure (9). Most of the members contain a single motif, except for uPAR, which contains three internal motifs, and the recently described snake serum-derived phospholipase A2 (PLA2) inhibitors, which contain two motifs (10, 11). The relationship of RoBo-1 to this family of proteins as well as its cellular localization and regulation suggests a novel role in bone metabolism.

EXPERIMENTAL PROCEDURES

RNA Isolation—RNA for PCR experiments was purified from tissues from 4-month-old female CD rats (Charles River Laboratories, Wilm-
Briefly, frozen tissues were pulverized under liquid nitrogen using a mortar and pestle and transferred to a 50-ml polypropylene tube. Immediately after the nitrogen evaporated, 10 ml of guanidinium thiocyanate solution containing 1 μl of antifoam A (Sigma) was added, and the homogenate was vortexed with a Polytron (Kinematica, Luzern) at high speed. Homogenates were transferred to 15-ml Corex tubes and centrifuged at 10,000 × g for 15 min to remove particulates. The supernatant was removed and transferred to a 50-ml tube. RNA was isolated according to manufacturer’s directions.

mRNA was isolated from total RNA using oligo(dT) spun column kit from Pharmacia Biotech Inc. The mRNA was double-selected according to the protocol.

Differential Display PCR — Total RNA from tibia, kidney, spleen, and brain was treated with RNase-free DNase using the Message Clean Kit from GenHunter Corp. (Brookline, MA) before DD-PCR. For DD-PCR, RNA from whole tibia, which included the peristome, marrow, and epiphyses, was used. PCR was performed according to the GenHunter kit with the following modifications. Three separate reverse transcription reactions containing 0.2 μg of RNA was performed for each 3′ primer. These reactions were pooled to reduce variability in this step. For each arbitrary primer, duplicate PCR reactions were performed using 2 μl of the pooled reverse transcription reaction/20-μl reaction. Samples were thermocycled 40 times as follows: 30 s at 94 °C, 2 min at 40 °C followed by a final extension at 72 °C for 5 min. 10 μl of the PCR products were diluted with 1 μl of 10 × gel loading buffer and analyzed on a 6% nondenaturing Tris-borate-EDTA gel. Gels were dried and exposed to x-ray film (X-Omat, Eastman Kodak Co.) over-night. Reproducible bands were excised, extracted, and reamplified according to instructions. Reamplified PCR fragments were subcloned into pGEM-T vector (Promega Madison, WI). Plasmid DNA was prepared using the Qiagen (Chatsworth, CA) miniprep kit.

Sequencing — AmpliTag dyeoxy sequencing was performed on an Applied Biosystems (Foster City, CA) ABI373A automated sequencer. Three independent full-length clones and one truncated clone were sequenced bidirectionally. Sequences were analyzed by Sequencher software (Gene Codes Corp.), and data bases searches were performed by both BLAST and FASTA algorithms. A novel, bone-specific cDNAs. One of these novel cDNAs, which was cloned into the XbaI site of pBluescript II KS(−) (Stratagene). The vector was linearized with NotI and transcribed with T3 polymerase in the presence of [35S]-UTP to generate the antisense probe. The sense strand was generated by linearizing with HinflIII and transcribing with T7 polymerase. Mid-coronal sections of the vertebrae 6 μm thick were cut and mounted onto glass slides coated with 3-aminopropyltriethoxysilane (Sigma). Prehybridization was performed as described previously (14). Sections were hybridized against radiolabeled probe (5 × 106 cpm/μl) for 16 h at 45 °C in a humidified environment. Sections were rinsed 2 times with 2 × SSC at room temperature, then incubated with ribonuclease A (50 μg/ml, Sigma) for 30 min in 2 × SSC at 37 °C. Sections were submitted to two more 1 × SSC washes at room temperature before final wash in 0.5 × SSC at 50 °C. The sections were dehydrated in graded alcohols containing 0.3 μl ammonium acetate and air-dried. For autoradiography, sections were coated with nuclear emulsion K5 (Ilford, UK). After a two week exposure, slides were developed, counterstained with hematoxylin and eosin, and mounted.

RESULTS

To identify mRNAs expressed exclusively in rat bone, we compared DD-PCR patterns from whole tibia to those of kidney, spleen, and brain. These tissues allowed us to eliminate potential common mRNAs from calcium-responsive, hematopoietic, and neuronal tissues respectively. Using a limited number of primer sets, we isolated 20 DD-PCR bands that were only visible in the lane containing bone RNA. After subcloning, sequencing, and Northern blot analysis, only two were novel, bone-specific cDNAs. One of these novel cDNAs, which we named RoBo-1, hybridized to a 1.6-kilobase mRNA that was expressed abundantly in bone yet showed little or no expression in other tissues (Fig. 1A, top blot).

Using the cloned PCR product as a probe, we isolated three identical 1,607-bp full-length cDNA clones from a rat tibia cDNA library. Northern blot analysis of rat tissues using this full-length cDNA probe confirmed the tissue distribution of RoBo-1 (Fig. 1A, middle blot). We also found RoBo-1 to be expressed in calvarial bone, cortical vertebra, tibial marrow, and diaphyseal bone denuded of marrow and periosteum (Fig. 2).
Furthermore, RoBo-1 was expressed in primary rat osteoblast cells isolated by sequential collagenase digestion (Fig. 1B) (12). Surprisingly, Northern blot (Fig. 1B) and PCR analysis (not shown) did not show expression of RoBo-1 in the osteoblast-like cell lines ROS 17/2.8 and UMR106. In addition, no expression was observed in the fibroblastic (Rat 1), the prostatic (MLL), nor the chondrocytic (IRC) cell lines (Fig. 1B) (15). These data suggested that the bone-specific expression of RoBo-1 is further restricted to specific cell types or differentiation stages.

The RoBo-1 cDNA contains a 110-bp 5'-untranslated region, an open reading frame of 720 bp coding for 240 amino acids (Fig. 2), and 777 bp of 3'-untranslated bases. This 3'-untranslated region sequence contained one potential polyadenylation signal and a rodent B2 repetitive element (16). After removal of this repetitive element, we searched the DNA databases and found no significant homology of the RoBo-1 cDNA to known sequences. Searching for protein homologies using the BLASTP algorithm revealed a significant homology of RoBo-1 to the 25 kDa (24% identity) and 30 kDa (26% identity) chains of the PLA2 inhibitor from monocled cobra Naja naja kaouthia (17). Using the FASTA algorithm, the strongest homology was found to be to a related PLA2 inhibitor protein, the Crotalus neutralizing factor from the South American rattlesnake Crotalus durissus terrificus (10). Similarity of RoBo-1 to these proteins was estimated to be approximately 45–47% (18).

Results of data base searching also revealed homology of RoBo-1 to the urokinase plasminogen activator receptor (uPAR). This correlation was significant since the snake PLA2 inhibitors and uPAR belong to a structurally related family of proteins that include uPAR, CD59, Ly-6, and snake venom neurotoxins (9). Although these proteins have diverse functions, the family is characterized by a common cysteine-rich domain of 70–90 amino acids containing 8–10 regularly spaced cysteines capable of forming disulfide bonds (Fig. 3) (19). All of the mammalian members of this family contain a single 90-amino acid cysteine-rich domain with the exception of uPAR.
which has three sequential domains. Based on the cysteine spacing, RoBo-1 likely has two of these cysteine-rich domains similar to the snake PLA2 inhibitors, to which it has the closest homology. The first such domain (amino acids 29–108) contains 10 cysteine residues as well as the conserved family motif CXXXXXCN, whereas the second domain (amino acids 127–195) has 8 cysteines, similar to the snake toxins. RoBo-1 is the first mammalian protein identified to have two sequential uPAR/CD59/Ly-6-like domains.

The computer program PSORT (Prediction of Protein Localization Sites) (20) suggested that the 21 hydrophobic residues at the N terminus could function as a signal sequence indicative of a secreted or membrane-associated protein. PSORT also predicted that RoBo-1 could be a type 1 membrane protein containing a short, hydrophobic trans-membrane domain made up of residues 218–234, which would leave a cytoplasmic tail of only six amino acids. However, as an alternative to membrane insertion, PSORT revealed the presence of a glycosylphosphatidylinositol linkage site, which suggested a potential covalent attachment to phosphatidylinositol on the cell membrane similar to other mammalian uPAR/CD59/Ly-6-like domains.

We verified that the RoBo-1 cDNA could serve as a template for protein synthesis using in vitro translation in both rabbit reticulocyte (Fig. 4) and wheat germ extracts (not shown). These experiments revealed a predominant protein of 25–30 kDa, consistent with the predicted open reading frame of 26.2 kDa (Fig. 4, lane 1). The protein sequence contains four possible N-linked glycosylation sites. Co-translation in the presence of canine microsomal membranes resulted in post-translation modifications that increased the molecular mass to 37–40 kDa (Fig. 4, lane 2). Treatment of extracts containing this modified RoBo-1 with peptide-N-glycosidase F (21) reduced the molecular mass of the translated product to the original size (Fig. 4, lanes 3–5), which verified that RoBo-1 has at least one functional N-linked glycosylation site.

To further localize the expression of RoBo-1 within bone, we performed in situ hybridization on longitudinal sections of rat caudal vertebrae. RoBo-1 cDNA showed labeling of the epiphyseal chondrocytes, where cartilage is resorbed and replaced by bone, as well as cells in the primary spongiosa (see Fig. 5, panels A (low magnification) and B (higher magnification)). This area is responsible for bone growth, and impairment of its function is associated with reduced bone volume (22). There was no labeling observed when the corresponding sense probe was used for hybridization (Fig. 5, panel C). As mentioned above, expression of RoBo-1 mRNA was not seen in the immortalized rat chondrocyte cell line (IRC) or in two chondrocyte-containing tissues, trachea (Fig. 1B) and ear (not shown). This specific expression of RoBo-1 in the epiphyseal chondrocytes and not in other cartilagenous tissues suggests that RoBo-1 is involved in forming mineralized tissue and may provide a marker for differentiation stages of chondrocytic cells.

Since RoBo-1 is expressed in areas of bone lengthening and remodeling, we were interested in the regulation of the RoBo-1 mRNA in response to a known bone formation stimulus. The strength at each site in the human skeleton is determined primarily by local mechanical forces placed on the bone. Mediators such as estrogen, parathyroid hormone, or calcitonin also have substantial effects on bone metabolism and bone mineral density. However, mechanical loading provides the critical signal that leads to formation of bone with the appropriate site-specific strength, density, and architecture. We examined in vivo regulation of RoBo-1 mRNA using two well-characterized mechanical loading models capable of producing strains that induce bone formation.

In the rat tail vertebral compression model, the eighth caudal vertebra is cyclically compressed along its length to induce bone formation (7). The vertebrae were subjected to a single episode of axial loading at strains normally experienced by bones during use. After this intermittent mechanical loading, we observed a reproducible increase in the RoBo-1 in situ hybridization signal in both the growth plate and primary spongiosa. This signal reached a maximum by 6 h (Fig. 5, panels D (low magnification) and E (higher magnification)) and returned to base line 24 h after loading. No labeling was observed after hybridization with the sense probe (Fig. 5, panel F).

In the axial ulna loading model (8), rat ulnae are compressed with intermittent mechanical forces that cause flexing at the weakest point along the bone. To adapt to these forces, new bone is formed at the site of deformation. Six h after this axial mechanical loading, RoBo-1 mRNA expression increased in the diaphysis of loaded ulnae compared with that of the contralateral control ulnae (Fig. 6). When densitometric values for RoBo-1 were normalized for β-actin mRNA expression, the level of RoBo-1 mRNA expression was increased more than 2-fold relative to that seen in the control bones (control = 0.30 ± 0.06, loaded = 0.63 ± 0.09, p = 0.035). The data from the two in vivo loading models implies that RoBo-1 may provide an important function in the ability of the bone to adapt to external forces applied to the skeleton.
Estrogen is known to have effects on bone mass, and removal of estrogen by ovariectomy results in the rapid loss of bone in rats (23). This loss can be prevented by restoration of estrogen. Since estrogen regulates bone metabolism and has been shown to alter the mRNA levels of known bone matrix proteins (24), we examined the effects of estradiol on RoBo-1 mRNA levels in ovariectomized rats. A single oral administration of estradiol (30 μg/kg) resulted in increased RoBo-1 mRNA levels in the tibial metaphysis over that of the nonloaded contralateral ulnae (Fig. 7). No hybridization was observed when the corresponding sense probe was used (panels C (control) and F (loaded)). Vertebrae were processed 6 h after mechanical stimulation (magnification: A and D, × 30; B, C, E, F, × 120).

**Fig. 5.** Localization of RoBo-1 by in situ hybridization; increase in RoBo-1 mRNA levels in rat vertebrae after mechanical loading. Sections from caudal vertebra from control rats (panels A and B) and those that had been mechanically stimulated with the tail compression model (panels D and E) were hybridized against a RoBo-1 antisense probe (bp 886–69). The nonstimulated vertebra show hybridization over ephiphyseal chondrocytes and over surfaces of primary spon- gia in growth plate cartilage. The signal increased when vertebra were subjected to a single 5-min episode of axial loading at 1 Hz to produce a peak strain of 770 microstrain (panels D and E). No hybridization was observed when the corresponding sense probe was used (panels C (control) and F (loaded)). Vertebrae were processed 6 h after mechanical stimulation (magnification: A and D, × 30; B, C, E, F, × 120).

**Fig. 6.** Increased expression of RoBo-1 mRNA in the ulna after mechanical load. Left ulnae of female Sprague-Dawley rats were subjected to mechanical stimulation in the nonsurgical axial loading model as described under “Experimental Procedures.” Six h after loading, animals were sacrificed, and ulnae were removed. Each lane represents pooled RNA from 4 ulnae. Results from four separate experiments are shown. Northern blots were carried out using 15 μg of total RNA/lane. Blots were hybridized using RoBo-1 cDNA (69–886 bp) or β-actin. Results from the loaded ulnae (Load) were compared with those from the nonloaded contralateral ulnae (Control).

**Fig. 7.** Increased expression of RoBo-1 mRNA in tibial metaphyses after oral administration of estradiol. Ovariectomized rats were dosed orally with a single 30 μg/kg dose of estradiol (E) or vehicle (V) as described under “Experimental Procedures.” Whole tibial metaphyses were harvested 24 and 48 h after dosing. RNA was prepared and Northern blotted as described under “Experimental Procedures” using 30 μg of total RNA/lane.

**DISCUSSION**

We described here the discovery of the novel bone-specific protein, RoBo-1, that is abundantly expressed in rat bone mRNA. Based on its sequence homologies, RoBo-1 is a new member of the uPAR/CD59/Ly-6 snake toxin family. These proteins contain a fundamental structural unit of approximately 90 amino acids that includes 8–10 cysteines that form internal disulfide bridges. Of interest is the fact that all of the mammalian members to date have only one of these structural units, with the exception of uPAR, which contains three. Also, studies have shown that these proteins share a common genomic organization (25). The 90 amino acid structural unit of the single domain proteins is coded for by two exons. This suggests that uPAR arose from a common ancestral gene likely resulting from internal triplication of the two exons. Since RoBo-1 has two cysteine structural domains, it likely has a similar gene organization resulting from internal gene duplication of similar ancestral exons. In fact, preliminary PCR analysis of rat genomic DNA suggests that RoBo-1 has an exon structure like other members of this family.

The closest homology of RoBo-1 is with the snake PLA2 inhibitor. These inhibitor proteins appear to have two cysteine domains, similar to RoBo-1. The Naja Naja PLA2 inhibitor functions as a heterotrimer composed of two 30-kDa subunits and a single 25-kDa subunit to inhibit PLA2. The Crotalus neutralizing factor PLA2 inhibitor functions as a oligomeric aggregate of 6–8 subunits to inhibit the actions of the PLA2 on cellular membranes. Although it is not known if RoBo-1 can inhibit PLA2, it is interesting to speculate that RoBo-1 may form functional homodimer, heterodimer, or oligomeric structures.

To date, none of the known uPAR/CD59/Ly-6 members have been found localized in a specific tissue. However, RoBo-1 mRNA expression was detected selectively in bone tissue and in growth plate cartilage. It is significant that RoBo-1 is expressed at sites of endochondral and membranous bone formation. Endochondral bone is formed in the growth plate by resorption of the calcified cartilage and replacement with bone. On the other hand, intramembranous bone is formed by osteo-
blasts without a prior cartilage template. Since RoBo-1 is expressed in osteoblast and growth plate chondrocytes but not in other cartilage tissue such as trachea and ear, it is likely to be involved in the bone formation functions of these two different cell types.

Both estrogen and intermittent mechanical stimulation are known to have net positive effects on bone density. Furthermore, it has been demonstrated that estrogen modulates the ability of the skeleton to respond to mechanical forces (26). RoBo-1 mRNA levels increased after estrogen replacement following ovariec-tomy. Similar increases were seen in bones after mechanical stimulation. It is significant that we observed increases after mechanical stimulation at sites of both endochondral (growth plate) and membranous bone formation (diaphysis). Since RoBo-1 is up-regulated when bone is formed through two different mechanisms, it suggests that RoBo-1 is critical to the ultimate formation of mineralized tissue.

Finally, the increased expression of RoBo-1 after mechanical stimulation and its homology to the snake PLA2 inhibitors is of particular interest for a number of reasons. After mechanical stimulation, there is a rapid, local increase in production of prostaglandins, which are potent regulators of bone formation and resorption (27, 28). Since a key regulator of prostaglandin synthesis is PLA2, a highly tissue-restricted inhibitor could provide a mechanism for local control of prostaglandin-mediated bone formation. Another possible mechanism could be to regulate the PLA2 activity associated with the extracellular matrix vesicles produced by chondrocytes and osteoblasts (29, 30). These matrix vesicles play an important role in the mineralization process. Although the function of RoBo-1 remains to be elucidated, its protein homologies, abundant mRNA expression, tissue specificity, and response to positive mediators of bone density suggest that it provides a unique function in the growth or remodeling of bone.

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