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Decorin is Processed by Three Isoforms of Bone Morphogenetic Protein-1 (BMP1)

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Abstract

The secreted small proteoglycan, decorin, modulates collagen fibril formation as well as the bioactivity of various members of the transforming growth factor- β (TGF β) superfamily. Indeed, recombinant prodecorin has been used in several gene therapy experiments to inhibit unwanted fibrosis in model diseases of the kidney, heart, and other tissues although the status of the propeptide within the target tissues is unknown. Currently the protease that removes the highly conserved propeptide from decorin is unproven. Using a variety of approaches, we show that three isoforms of the Tolloid-related bone morphogenetic protein-1 (BMP1) can effectively remove the propeptide from human prodecorin resulting in the well-established mature proteoglycan. Classic BMP1, the full-length gene transcript mTLD (BMP1-3), and BMP1-5 (isoform lacking the CUB3 domain thought to be important for efficient type I collagen C-propeptidase activity) all removed the analogous propeptides from both recombinant human prodecorin and murine probiglycan. Furthermore, the timed removal of the propeptide was found to not be necessary for the addition of decorin's single glycosaminoglycan chain. Decorin therefore joins the growing list of matrix and bioactive molecules processed/activated by the BMP1/Tolloid family. Since the third member of the Class I small leucine-rich proteoglycan (SLRP) superfamily, asporin, also contains a similar cleavage motif at the appropriate location, we propose that the removal of these propeptides by members of the BMP1 family is an additional characteristic of Class I SLRP.

Keywords

Decorin; Biglycan; BMP1; mTLD; BMP1-5

Introduction

¹PG40 from a human embryonic fibroblast cell line was the first small proteoglycan (PG) to be cloned [1], although the name was later changed to decorin (DCN) to reflect that the protein appeared to "decorate" collagen fibrils in electron micrographs [2]. Decorin cDNA encodes a classic leader sequence immediately followed by a highly conserved 14-amino acid prodomain

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¹The abbreviations used are: PG, proteoglycan; DCN, decorin; BGN, biglycan; SLRP, small leucine-rich proteoglycans; TGF β , transforming growth factor- β ; BMP1, bone morphogenetic protein-1; mTLD, mammalian Tolloid; cABCase, chondroitinase ABC; CUB domain, protein domain first found in the complement components C1r/c1s, the sea urchin protein Uegf, and BMP1; mTLL, mammalian Tolloid-like; hBMSC, human bone marrow stromal cells; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PCPE, procollagen C-proteinase enhancer.

that must be removed to produce the “mature” form of proteoglycan characteristic of those isolated from a variety of tissues (e.g., placental membranes, skin, bone, and cartilage). To illustrate the conservation of the DCN propeptide, 12 of the 14 amino acids remain identical [(G/K)PF(Q/H)QRGLFDFMLE] between humans and reptiles (Anoli). Later, bone matrix was shown to contain both DCN (with a modified serine at the predicted GAG attachment site) and a second small PG that was distinguished from DCN by both its amino-terminal sequence and immunoreactivity [3]. The second small PG showed significant homology to DCN in the leucine-rich repeat domain first proposed by L. Patthy [4] as well as in the conserved locations of both amino- and carboxy-terminal cysteine clusters [5]. In addition, it contained a prodomain similar to that found in DCN and two GAG chains near the mature protein’s aminoterminal, hence it was named biglycan (BGN). In 2000, the Tolloid-related protease, bone morphogenetic protein-1 (BMP1), was shown to efficiently remove the prodomain of human proBGN [6], but the protease for proDCN has not been directly proven.

The expression patterns of these two Class I small leucine-rich proteoglycans (SLRP) are divergent and often mutually exclusive. DCN was found within all major type I and II collagen matrices while BGN was found in a range of specialized tissues (e.g., myofibers and endothelial cells) and epithelial cells (e.g., immature keratinocytes, and renal tubular epithelia) [7]. DCN binds to the “holes” or gaps in the surface of fibrils resulting from the staggered assembly of mature collagen trimers. Mice lacking DCN have irregular collagen fibrils and fragile skin suggesting that this PG may be involved in collagen fibril assembly [8]. DCN has also been shown to bind to transforming growth factor- β (TGF β), neutralizing its bioactivity [9]. Other members of the TGF β superfamily also appear to bind to DCN and BGN [10]. It has been proposed that TGF β members may also be stored in various matrices by binding to SLRP/collagen complexes. Due to its ability to bind TGF β , recombinant DCN has been used in several gene therapy applications. For example, de novo expression of proDCN in muscle prevented fibrotic disease in experimental rat glomerulonephritis [11], and more recently proDCN expression mitigated cardiac fibrosis in both spontaneously hypertensive rats [12] and ligation-induced myocardial infarction [13]. Although most data support DCN’s role in the negative modulation of TGF β -related activities, one paper showed that DCN-null myoblasts had aspects of their TGF β signaling response restored upon reintroduction of proDCN expression [14]. Others have shown that DCN enhanced the binding of TGF β to its receptors thereby enhancing bioactivity [15]. Importantly, it is not known if the highly conserved propeptide is removed during the biogenesis of DCN in the transduced tissues of these gene therapy protocols or which form (proDCN or mature) may be more effective in modulating such TGF β activities. Thus, knowledge of the proteases involved in the removal of the proDCN propeptide is important for our basic understanding of this conserved PG and may enable more targeted results in future gene therapy studies.

Materials and methods

Cell Culture

The human embryonic kidney cell line (HEK293A) was obtained from Invitrogen (Carlsbad, CA) and maintained in Dulbecco’s modified Eagle Medium (Gibco-BRL, Gaithersburg, MD). Primary human bone marrow stromal cells (hBMSC) (gift from Drs. Pamela Gehron Robey and Sergei Kuznetsov, NIDCR/NIH) were grown in α -minimum essential medium (Gibco-BRL) supplemented with 20% fetal bovine serum except as indicated. All media were supplemented with L-glutamine (2 mM), penicillin (100 IU/ml), and streptomycin (100 μ g/ml).

Antibodies

Polyclonal goat anti-human BMP1 antibody (AF1927) was from R&D Systems (Minneapolis, MN). Rabbit polyclonal antibodies against mature mouse biglycan (LF-159), the propeptide domain of human biglycan (LF-104), mature human decorin (LF-136), and the human propeptide domain of decorin (LF-111) were previously published [16]. IRDye 680 donkey anti-goat and IRDye 680 goat anti-rabbit IgG second antibodies were from LI-COR Biosciences (Lincoln, NE).

Recombinant Proteins

Human prodecorin and mouse probiglycan-encoding replication-deficient adenoviruses were made by inserting full length cDNAs (clone P2 and mDCN-4 respectively, [16]) into adenovirus constructs and selecting for appropriately expressing adenovirus plaques in HEK293 cells. HBMSC were transduced with adenoviruses and cultured for 48 h before the addition of fresh serum-free medium containing 100 μ M of synthetic furin inhibitor, dec-Arg-Val-Lys-Arg-chloromethylketone (Enzo Life Sciences, Plymouth Meeting, PA). Proteoglycans were purified from the conditioned medium on Q Sepharose Fast Flow anion exchange columns (Amersham/Pharmacia, Uppsala, Sweden) using NaCl step gradients. Recombinant human BMP1–5 was generated as described previously [17]. Recombinant mouse mammalian tolloid (mTLD) was made by transferring the full length cDNA from clone ID6849066 (Invitrogen, Carlsbad, CA) first into the Gateway pENTR plasmid and subsequently into pAD/CMV/V5-DEST to make the replication-deficient adenovirus according to the manufacture's protocol (Invitrogen). Human BMP1 (catalog # 1927-ZN) was purchased from R&D Systems. Recombinant BMP1–5 and mTLD proteins were obtained from the conditioned serum-free medium of transduced hBMSC. The three isoforms of recombinant BMP1 proteins were verified by Western blot using the goat anti-human BMP1 (catalytic domain) antibody. Conditioned media from nontransduced hBMSC had no detectable BMP1-like activity under the conditions used in the assays described below.

In vitro cleavage assays

Samples of purified human prodecorin dissolved in reaction buffer (25 mM HEPES, pH 7.5, 5 mM CaCl_2 , 0.01% Brij) and divided into aliquots containing 2 μ g each and aliquots of serum-free media containing mouse probiglycan dialyzed against reaction buffer were each incubated at 37°C for 19 h alone or in the presence of indicated BMP1 isoforms. All samples were also digested with 2.5 mU of chondroitinase ABC (cABCCase; Seikagaku America Inc., Ijamsville, MD) followed by addition of manufacturer's concentrated buffers and denaturants for treatment with 2.5 mU of N-Glycanase (PNGase F, ProZyme, San Leandro, CA) for 3 h at 37°C. Proteins were then denatured with reducing SDS sample buffer, electrophoresed on 10% Tris-Glycine gels (Invitrogen), and transferred to Immobilon-FL membrane (Millipore, Billerica, MA). Blots were blocked for 1 hr in Odyssey Blocking Buffer (LI-COR Biosciences), followed by incubation in a 1:1,000 dilution of the noted polyclonal rabbit antibodies overnight at 4°C. After three washes of 5 min each in PBS containing 0.05% Tween-20, blots were incubated in a 1:10,000 dilution of IRDye goat anti-rabbit IgG for 1 hr and then given a final 3 washes. All latter steps were at room temperature. Proteins were visualized using the LI-COR Odyssey infrared imaging system (LI-COR Biosciences). SeeBlue Plus2 Pre-Stained Standards (Invitrogen) were used as protein standards.

Amino Acid Sequence Analysis

10 μ g samples of purified recombinant prodecorin were incubated with and without 300 ng of recombinant BMP1 and 10 mU of cABCCase at 37°C for 19 h. Protein samples were then treated with 2.5 mU of N-Glycanase (PNGase F), electrophoresed on a SDS 10% Tris-Glycine gel, and electro-transferred onto an Immobilon-FL membrane. Coomassie blue-stained bands

corresponding to the core proteins were microsequenced by automated Edman degradation at the Facility for Biotechnology Resources (CBER, FDA, Bethesda, MD) using the Procise ABI Model 494A Protein Sequencing System.

Results and discussion

The presence of a decorin propeptide was evident from the initial cloning of this widely expressed small proteoglycan, but it is so efficiently removed *in vivo* that it is rarely detected even in extracts of DCN-rich whole tissues [18]. This lack of a full-length proDCN substrate has hindered the search for definitive proof of the protease responsible for the removal of this highly conserved domain. From the work of Scott et al. [6], we know that another Class I SLRP, human proBGN, is cleaved at various efficiencies by BMP1/Tolloid family members. Because 1) BMP1-null mice die before birth, 2) cell lines from these mice are not publically available, and 3) even if such cells were used to make proDCN, the two other BMP1-like genes (mTLL1 and mTLL2) may be sufficient to process significant amounts of proDCN and proBGN. Therefore, the processing of proDCN is best studied with PG isolated from cells lacking activity for all BMP1/mTLL gene products. Knockdown of all three gene products by siRNA (or similar technologies) are usually not 100% efficient and protease activity remaining from such approaches could be sufficient to process the proteins. Therefore, we chose to make the proPGs in cells that lack the ability to activate the BMP1/mTLL proteases. Furin is known to activate the BMP1/mTLL proteases at a well-defined site [19]. In the past we have used the furin-deficient human colon carcinoma cell line, LoVo, to make other glycoproteins that are also cleaved by BMP1 family members, but our preliminary work suggested that LoVo cells were inefficient at biosynthesizing the GAG chains on DCN (data not shown). Fortunately, we were able to purify proDCN and proBGN proteoglycans by transducing primary human bone marrow stromal cells capable of placing GAG chains on proDCN and proBGN in the presence of a synthetic furin inhibitor that effectively enters cells. This furin-inhibitor causes BMP1/Tolloid proteases to remain inactive and therefore unable to process their normal substrates. For example, type I collagen in the furin-inhibited conditioned media were observed as procollagen bands on PAGE (data not shown). Figure 1A shows that the proDCN proteoglycan did not co-purify with any DCN protein lacking its single highly charged GAG chain (lane 1). Upon digestion with chondroitinase ABC, the well-known DCN doublet core protein appeared at the expected M_r (lane 2) and that this doublet core protein was then converted to a single, lower M_r protein band after removal of its N-linked oligosaccharides by N-glycanase (lane 3). Because both proDCN and proBGN purified by anion exchange chromatography had their GAG chains attached, it is clear that the initiation and elongation of these chains is not dependent on the removal of the propeptide as once proposed [20]. Our results also complement the work of Oldberg et al. [21] in which they showed that deletion of most of the rat DCN's propeptide also did not result in the loss of GAG chain addition in cell culture experiments. Therefore, neither the presence nor appropriately timed removal of their respective propeptides is necessary for GAG chain addition to DCN or BGN.

The human proDCN (and mouse proBGN used as a positive control) PG purified from the furin-inhibited cell culture media were then digested with recombinant classic BMP1 (BMP1-1) to test for the removal of propeptides. For the clearest western blot analyses, both GAG and N-linked oligosaccharide chains were also removed from the BMP1-treated samples by chondroitinase ABC and N-glycanase respectively prior to SDS-polyacrylamide gel electrophoresis and immunodetection. For clarity, the leader and propeptide sequences as well as the peptides synthesized to make the four antibodies used for analyses are noted in Figure 1B. Figure 2A (left panel) shows that treatment of purified proDCN PG with recombinant BMP1 effectively removed its propeptide as seen by the loss of the propeptide-positive immunodetected band. The remaining core protein is observed in the right panel (using the more C-terminal antibody) as a consistently faster electrophoresed core protein, due to the loss

of its 14 amino acid propeptide (~1.7 kDa). In a control experiment paralleling the results of Scott et al. [6] for human proBGN, treatment of the purified mouse proBGN PG with classic BMP1 also resulted in the loss of its propeptide band (using BGN propeptide antibody, Figure 2B, left panel). A M_r shift caused by the loss of BGN's ~2.6 kDa propeptide when detected by BGN's more C-terminal antibody is seen in Figure 2B, right panel.

While loss of DCN's propeptide epitope and retention of its "mature" epitope was indicative of BMP1's cleavage at the expected M ϕ X-D motif, N-terminal protein sequencing remains the gold standard. The amino terminus of the proDCN was GPFQQ proving for the first time that the most commonly predicted cleavage site for removal of the leader sequence is what is actually produced in cells (hBMSC). The microsequence of the DCN core protein after digestion with recombinant BMP1 was DEAXGIGPEV verifying that this Tolloid-related protease did cleave at the expected motif and resulted in the sequence identical to that reported for naturally occurring, mature human DCN [22]. The inability to detect the serine encoded at amino acid position number 4 (X) of the BMP1-treated protein also verified that the PG made for these experiments was chemically modified (logically by the addition of the GAG chain) at this protein's predicted GAG-attachment motif.

BMP1 is the most commonly studied isoform but others such as the full-length gene transcript, mTLD (BMP1-3), and the shortest isoform to retain an intact catalytic domain, BMP1-5, are also commonly expressed in a variety of tissues [23]. Indeed, it has been proposed that a combination of the BMP1 isoforms and enhancer proteins (e.g., PCPE-1 and PCPE-2) expressed by a cell/tissue that directs the processing of the many matrix and other bioactive proteins that are known to be activated/processed by BMP1 [24]. Scott et al. [6] showed that BMP1 effectively removed the propeptide from human proBGN while the longest isoform, mTLD, showed barely detectable activity under their conditions. Under our experimental conditions recombinant BMP1, mTLD, and BMP1-5 all effectively processed both human proDCN (Figure 3A) and murine proBGN (Figure 3B) as measured by both the loss of their respective propeptide epitopes (left panels) and the slightly decreased apparent molecular weight of their respective mature core proteins (right panels) on western blots. While our more complete processing of the murine proBGN by mTLD compared to the previous authors' human proBGN substrate [6] could be due to the species difference, it is more likely that our more enabling digestion condition is the explanation. The processing of proDCN and proBGN by BMP1-5 isoform reported here for the first time is interesting because this shortest of proteolytically active isoforms lacks the CUB3 domain thought to be important for the successful binding and cleavage of the prototypical BMP1 substrate, type I procollagen [25]. In our hands, BMP1-5 remains a much less efficient processor of type I procollagen than BMP1 (data not shown), but it does remove the propeptides of proDCN and proBGN thereby indirectly supporting the previous hypothesis that the CUB3 domain may be directed towards the processing of collagen substrates and not the Class I SLRPs. Similarly, we agree that mTLD, which was recently reported to dimerize in the presence of ionic calcium and become inefficient at processing type I procollagens [26], was inefficient at processing human procollagen *in vitro* (data not shown) but it was relatively efficient at removing the propeptides of DCN and BGN. This supports the hypothesis that mTLD and BMP1-5 may have been selected for processing noncollagenous substrates *in vivo*.

In this report we have shown that a second member of the Class I SLRPs, decorin, to have its propeptide removed by isoforms of BMP1. The predicted coding sequence of the third member of this class, asporin, also contains a similar propeptide [27] that can reasonably be proposed to be removed by BMP1/Tolloid-related proteases at its own M ϕ X-D motif. Furthermore, if the observation that asporin is not a proteoglycan and yet is biosynthesized with a propeptide that is removed by the Tolloid-related proteases hold true, it offers another proof that the propeptide itself is not directly involved in the addition of the glycosaminoglycan chains. A

corollary note is that over the long period of evolution since these three genes began diverging, all three proteins continue to maintain their small propeptide suggesting that they continue to play an as yet unknown but important role in the biology of these secreted proteins.

Conclusions

Decorin's ability to bind to TGF β superfamily members has led to proDCN's use in blocking unwanted fibrosis in a number of model systems even though the enzyme that removes its highly conserved propeptide was unproven. Inhibition of the furin-activation of the BMP1/Tolloid members permitted the efficient production of the PG forms of human proDCN and mouse proBGN in virally transduced cells. The presence of GAG chains on the proPGs showed that the removal of the propeptide is not necessary for the addition of these chains. Three isoforms of BMP1 ranging from the shortest (BMP1-5, lacking the CUB3 domain important to processing procollagen) to the longest (mTLD, inefficient at processing procollagen in vitro) were all shown to be capable of removing the highly conserved propeptides from both proDCN and proBGN PG. Future studies are necessary to determine importance of the timing and location of the removal of these highly conserved propeptides in vivo and the roles that they may play in the bioactivity of the TGF β superfamily.

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Fig. 1. Detection of prodecorin core protein and illustration of relevant peptide sequences. (A) Western blot analysis of core protein region of human proDCN shows that removal of glycosaminoglycan chains with chondroitinase ABC (cABCCase) results in an observed classic double core protein (-/+) while subsequent removal of N-linked oligosaccharides by N-glycanase results in a single band (+/+). Intact proteoglycan is not transferred and results in an empty lane on western blot (-/-). Detection was with rabbit anti-human DCN peptide antibody as described in panel B. (B) Notation of the leader, propeptide, and first several amino acids of the human preprodecorin and murine preprobiglycan sequences. Boxed are the peptides used to generate the corresponding peptide antibodies in rabbits. Arrowheads indicate locations of glycosaminoglycan chain attachment, * indicates location of single amino acid difference between human peptide antigen sequence (r) used to make the LF-104 antibody and the murine sequence at that position (k).

Fig. 2.

The removal of propeptides by bone morphogenetic protein-1 (BMP1) from both proDCN and proBGN are indicated by both loss of propeptide epitopes and small reduction in M_r of remaining core proteins on western blots. (A) Left panel shows that the epitope within the propeptide of human proDCN is lost upon the treatment with BMP1 (+). The same products immunodetected with epitope located C-terminal to the glycosaminoglycan attachment motif shows the intact core protein differs in M_r due to loss of the propeptide (right panel). (B) The removal of the propeptide of mouse proBGN by BMP1 was proven by the loss of the propeptide epitope (left panel) and a shift detected by BGN's more C-terminal antibody upon treatment with BMP1 (+). All samples were treated with enzymes to remove glycosaminoglycan and N-

linked oligosaccharide chains prior to electrophoresis on 10% Tris/Glycine SDS polyacryamide gels. Antibodies used for detection were as indicated.

Fig. 3.

Three isoforms of BMP1 remove propeptide from both human proDCN and murine proBGN. (A) Human proDCN was digested with classic BMP1, full-length isoform (mTLD), or shortest active isoform that lacks the CUB3 domain (BMP1-5) and detected with either antibody to the propeptide (left panel) or mature protein (right panel). Note that all three isoforms cause the loss of the propeptide epitope and the small but reproducible reductions in M_r detected by the antibody that can detect both forms. (B) The propeptide of mouse probiglycan is also predominantly lost when treated with the same three isoforms of BMP1 as detected with propeptide antibody (left panel) and while mature protein antibody detection remains strong and slightly shifted to a lower M_r (right panel). All samples were treated with enzymes to

remove glycosaminoglycan and N-linked oligosaccharide chains prior to electrophoresis on 10% Tris/Glycine SDS polyacryamide gels. Antibodies used for detection were as indicated.