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The Production and Purification of Functional Decorin in a Baculovirus System[1]

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Human decorin was expressed in *Spodoptera frugiperda* 21 (Sf21) insect cells. A full-length cDNA encoding preprodecorin of 359 amino acids from a human fibroblast library was cloned into baculovirus transfer vector pVL1392, and transfected into Sf21 insect cells. The infected cells secreted the mature decorin into the culture medium. The secreted decorin lacked glycosaminoglycan but was N-glycosylated, whereas the unmodified decorin was present in the cell lysates, suggesting that N-glycosylation is required for decorin secretion from Sf21 cells. The recombinant decorin was then efficiently purified from the conditioned medium by two chromatographic procedures, hydroxyapatite Sepharose and Con A-Agarose, under nondenaturing conditions. The purified decorin was more potent, as evaluated by the inhibition of collagen fibrillogenesis, than that obtained from bovine tissues under denaturing conditions. The final yield of recombinant decorin was 1.5 mg in 200 ml culture medium of 3×10^8 cells. The biologically active decorin produced in Sf21 cells is a potentially useful probe for investigating the molecular interactions of this protein with other extracellular matrix proteins and may also have therapeutic applications.

The dermatan sulfate proteoglycan decorin, also known as PGII or PG40, is a ubiquitous component of the extracellular matrix (ECM) of various tissues (1,2). Decorin, together with biglycan, comprises a small-proteoglycan family characterized by a protein core of approximately 40 kDa containing 8-12 leucine-rich repeat sequences (3). Decorin is modified by a single glycosaminoglycan (GAG) chain near the N-terminus and is N-glycosylated at three potential sites (4). Decorin is capable of binding various ECM proteins *in vitro,* and has thus been suggested to play an important role in development and maintenance of the structural and functional integrity of organs (1,5). Binding to transforming growth factor-β (TGF-β) modulates this ubiquitous growth regulatory factor and is involved in cell proliferation and differentiation, and ECM accumulation (6,7). Binding to collagens inhibits fibrillogenesis (8,9), and that to fibronectin modulates fibronectin-mediated cell adhesion (10-12). On the basis of these activities, which prevent ECM deposition, decorin has recently been recognized as a promising therapeutic agent for fibrotic disorders such as glomerulonephritis (13,14).

Most of the decorin samples utilized to investigate these molecular interactions have been purified from connective tissues. Employing such preparations, a large quantity of decorin can be obtained only after solubilization of the homogenate with denaturing reagents, such as guanidine hydrochloride (Gdn HCl), which disrupt the higher-order or native structure of this protein (15,16). This treatment does not usually allow proper refolding of decorin after removal of the denaturant, as was suggested by impaired binding to type I collagen by decorin upon exposure to Gdn HCl (17).

In the present study, we produced a post-translationally modified decorin in a baculovirus expression system. Although the recombinant decorin was devoid of GAG, the N-glycosylated mature decorin was secreted in the culture medium and easily purified by a novel method under non-denaturing conditions. The purified decorin displayed a remarkable inhibitory effect on collagen fibrillogenesis.

MATERIALS AND METHODS

Isolation of human decorin cDNA and construction of transfer vector pVL1392-hDCN. A digoxigenin-labeled RNA probe for the coding region of human decorin was generated as described previously (18). A human fibroblast λgt10 cDNA library (Clontech, Palo Alto, CA) was screened with the probe, and the insert in the positive phage was subcloned into an EcoRI site of pBluescript II SK+ (Stratagene,

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La Jolla, CA). After verification of the sequence, the insert cDNA sequence was released from the plasmid by XbaI/EcoRV double digestion, and then ligated between the XbaI and SmaI sites in the multiple cloning site of the baculovirus expression vector pVL1392 (Invitrogen, NV Leek, The Netherlands).

Generation of a recombinant baculovirus. *Spodoptera frugiperda* Sf21 cells were grown in Grace's insect medium (Gibco BRL, Gaithersburg, MD) supplemented with 10 % fetal bovine serum, 0.33 % TC yeastolate (Difco, Detroit, MI), 0.33 % lactalbumin hydrolysate, 0.035 % sodium bicarbonate, and 50 μg/ml gentamycin at 27°C. To generate recombinant virus, 0.5×10^6 Sf21 cells were cultured in a 25 cm^2 tissue culture flask for 10 h. After replacing the medium with a serum-free medium, a 2 μg quantity of purified pVL1392-hDCN was cotransfected into the cells with 20 ng of BaculoGold DNA (Pharmigen, SanDiego, CA) using a Lipofectin reagent (Gibco BRL) (19). The recombinant virus was plaque-purified and amplified.

Production of decorin by Sf21 cells. A total of 3×10^8 Sf21 cells was seeded onto 20 plates of a 150 mm culture dish and incubated for 24 h. The medium was replaced with serum-free Sf-900 medium (Gibco BRL), and the cells were infected with the amplified recombinant virus at a multiplicity of infection (MOI) of 1.0. At 5 days post-infection, the conditioned medium and infected cells were harvested.

Purification of the recombinant decorin. First, 200 ml of conditioned medium were centrifuged at 5000 × g for 30 min. A 180 ml quantity of the supernatant was dialyzed against phosphate-buffered saline (PBS), then applied to a hydroxyapatite Sepharose column (1.5 × 15 cm) (Bio-Rad, Richmond, CA) equilibrated with the same solution. The column was washed with PBS ("wash1") followed by a solution containing 0.1 M potassium phosphate and 0.1 M NaCl at pH 6.8 ("wash2"). The crude decorin was eluted with 0.27 M potassium phosphate at pH 6.8. The 27 ml eluent was then diluted to 50 ml with distilled water, and applied to a concanavalin (Con) A-Agarose column (1.2 × 9 cm) (Seikagaku Kogyo, Tokyo, Japan) equilibrated with PBS. After washing the column with 20 mM α-methylmannoside in PBS, decorin was eluted with 0.2 M α-methylmannoside in PBS.

The N-terminal sequence of the purified recombinant decorin was analyzed using a model 473A protein sequencer (Perkin-Elmer, Foster City, CA). Removal of the N-linked oligosaccharides was carried out employing N-glycosidase F (Boehringer Mannheim, Germany).

SDS–PAGE and Western blotting analysis. The purified decorin, conditioned medium and cell lysates were analyzed on a 10 % SDS polyacrylamide gel under reducing conditions. The protein bands were visualized with Coomassie Blue R-250 or by Western blotting analysis using a rabbit anti-human decorin polyclonal IgG (Chemicon, Temecula, CA) followed by a horseradish peroxidase-conjugated antibody to rabbit IgG (Dako, Glostrup, Denmark). The immunoreactive bands were visualized with a chemiluminescence ECL kit (Amersham, Buckinghamshire, UK).

Inhibition of collagen fibrillogenesis. The collagen fibrillogenesis inhibition assay using decorin was carried out according to the method of Vogel *et al.* (20) with some modifications. Briefly, 50 μg of porcine skin type I collagen (Wako, Osaka, Japan) and decorin corresponding to 3 μg of the core protein were dissolved in 1 ml of solution containing 30 mM sodium phosphate and 0.14 M NaCl at pH 7.3, and incubated at 37°C. Self-aggregation of collagen was monitored by the absorbance at 405 nm.

Bovine decorin was purified from tendon under denaturing conditions (4 M Gdn HCl) as described previously (16). The Gdn HCl-treated recombinant decorin was prepared by incubating the recombinant decorin in a denaturing solution containing 4 M Gdn HCl and 50 mM Tris-HCl at pH 6.6 for 4 h at room temperature, and then the solution was exchanged for PBS using Ultrafree-MC tube (Millipore, Bedford, MA).

RESULTS

Expression of Human Decorin in Baculovirus-Infected Sf21 Cells

Three decorin cDNA clones were isolated from the 5×10^5 λgt10 phages screened. In these clones, the 5′ non-coding region corresponding to the first exon was derived from the newly identified exon 1c which was different from either of the known exons, 1a and 1b (21). The genomic structure of exon 1c will be described elsewhere. The coding sequence of these clones was identical to that reported previously (4).

The full-length decorin cDNA with 81 and 617 bases of the 5′ and 3′ non-coding regions, respectively, was cloned into the baculovirus expression plasmid pVL1392 behind the promoter of the polyhedrin gene. Sf21 cells were infected with high-titer recombinant baculovirus stock. When the 20 μl of the culture medium at 5 days post-infection was analyzed on SDS-PAGE, recombinant decorin with an approximate molecular mass of 43 kDa was detected by Coomassie Blue staining (Figure 1a, lane 2) and Western blotting analysis (Figure 1b, lane 2). The native decorin has a single glycosaminoglycan (GAG) chain, and yields a diffuse band migrating between 97 and 200 kDa (16). In contrast, as judged by the discrete electrophoretic pattern, the recombinant decorin was suggested to be devoid of GAG chains. The GAG deletion was confirmed by the absence of the molecular mass shift that should have

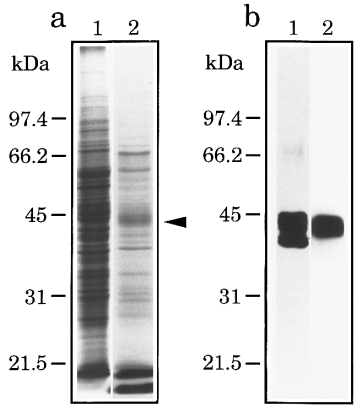

FIG. 1. Human decorin expressed in Sf21 cells. 1.5×10^7 Sf21 cells were seeded onto a 150 mm culture dish and infected with recombinant baculovirus at a multiplicity of infection of 1.0. After incubation for 5 days at 27°C, the conditioned medium (10 ml) and cells were harvested. a. 20 μg of the cell lysates (lane 1) and 20 μl of the conditioned medium (lane 2) were resolved on a 10 % SDS polyacrylamide gel under reducing conditions and stained with Coomassie Blue R-250. The position to which the recombinant decorin migrated on the gel is indicated by an arrow. b. 2 μg of the cell lysates (lane 1) and 1 μl of the medium (lane 2) were electrophoresed, followed by Western blotting analysis. The band at 37 kDa (lane 1) was the unglycosylated decorin core protein, as described in RESULTS.

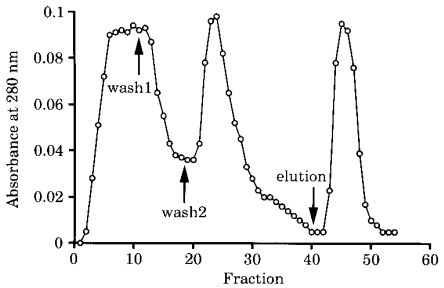

FIG. 2. Hydroxyapatite chromatography of the conditioned medium of infected Sf21 cells. At 5 days post-infection, the serum-free medium (180 ml) was applied to a 1.5 × 15 cm hydroxyapatite Sepharose column. Following the washing steps, fractions 43–51 eluted with 0.27 M potassium phosphate were collected. The fraction volume was 5 ml before No. 15 and 3 ml thereafter. The "wash1" and "wash2" steps were carried out utilizing PBS and a solution containing 0.1 M potassium phosphate and 0.1 M NaCl, respectively.

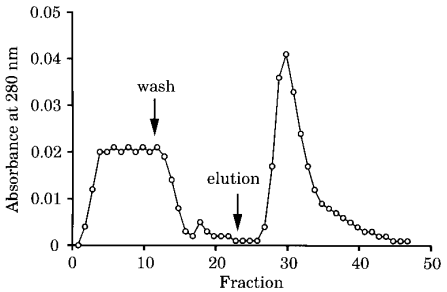

FIG. 3. Con A-affinity chromatography of the crude decorin sample. The 27 ml eluent of the preceding hydroxyapatite Sepharose chromatography was diluted to 50 ml with distilled water, and then loaded onto a 1.2 × 9 cm Con A-Agarose column. Following the washing step ("wash") using 20 mM α-methylmannoside in PBS, the recombinant decorin was eluted with 0.2 M α-methylmannoside as indicated by "elution". The fraction volume was 1 ml.

resulted from chondroitinase ABC digestion (data not shown). The expressed decorin was detectable by Coomassie Blue staining and Western blotting analysis in the cell lysates as well (lane 1 of Figures 1a and 1b).

Purification of Recombinant Decorin Produced in Insect Cells

Infected Sf21 cells were cultured in serum-free medium. In order to purify the recombinant decorin secreted into the medium, we tried various chromatographic modalities including ion-exchange, phenyl-Sepharose and blue-Sepharose, and finally devised a two-step chromatographic method using hydroxyapatite followed by Con A. Denaturing buffers were not utilized in these purification steps. A typical chromatogram using a hydroxyapatite Sepharose column is presented in Figure 2. It should be noted that the second washing, or "wash2", with a solution containing 0.1 M potassium phosphate and 0.1 M NaCl was indispensable for purification via the following Con A chromatographic step. The crude decorin was eluted with 0.27 M potassium phosphate.

Subsequently, the collected fractions were applied to a Con A-Agarose column (Figure 3). Decorin was tightly bound to the column during the washing with PBS followed by 20 mM α-methylmannoside, and finally eluted with 0.2 M α-methylmannoside. The rationale for the use of Con A is described below.

Characterization of the Recombinant Decorin

The decorin obtained from the Con A affinity chromatography was analyzed by SDS-PAGE. Coomassie Blue staining and Western blotting analysis revealed that the sample was pure and the molecular mass of the purified decorin was 40-43 kDa (Figure 4). When digested with N-glycosidase F, the purified recombinant decorin decreased to 37 kDa in molecular mass, indicating N-glycosylation. This finding also suggested that the band at 37 kDa observed in the Western blot of SF21 cell lysates (Figure 1b, lane 1) was the unglycosylated decorin core protein.

The decorin cDNA in pVL1392-hDCN encoded a leader sequence, which was comprised of the signal peptide of 16 amino acid residues and the succeeding putative propeptide of 14 residues (4), and the mature decorin. Sequence analysis revealed that the majority of the secreted recombinant decorin was in the properly processed mature form and that the prodecorin, which is also detected in the human articular cartilage (22), was present as a minor fraction comprising less than 20 % of the total decorin (data not shown).

The final yield of the purified mature decorin was 1.5 mg from 200 ml of culture medium containing 3 × 10^8 Sf21 cells.

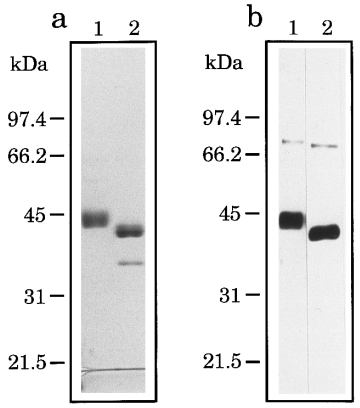

FIG. 4. SDS–PAGE of the purified recombinant decorin. The purified recombinant decorin before (lane 1) and after (lane 2) N-glycosidase F digestion was loaded onto a 10 % SDS polyacrylamide gel under reducing conditions. a. Coomassie Blue staining of 2 μg of decorin. The band at ~34 kDa is N-glycosidase F (lane 2). b. Western blotting of 20 ng of decorin. The protein bands observed at ~85 kDa (lane 1) and ~75 kDa (lane 2) were the dimeric decorin.

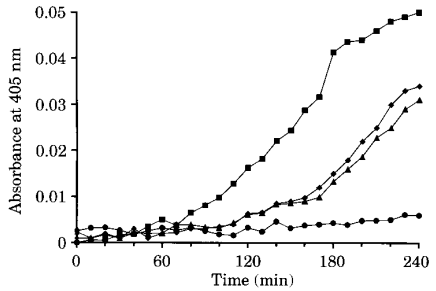

FIG. 5. Inhibition of collagen fibrillogenesis by various decorin samples. The fibril formation of 50 μg/ml solution of type I collagen was monitored in the presence of 3 μg/ml of the recombinant decorin (●), Gdn HCl-treated recombinant (▲), native decorin (♦), or a blank solution (■). Polymerization was monitored by the absorbance at 405 nm. The native decorin had been purified from bovine tendon under denaturing conditions (4 M Gdn HCl). The concentration of decorin was based on that of the core protein.

Inhibition of Collagen Fibril Formation by Recombinant Decorin

Inhibition of collagen fibril formation is the representative property of decorin *in vitro*. The recombinant decorin was evaluated with respect to this activity. Type I collagen monomers in a 50 μg/ml solution spontaneously form polymer fibrils when incubated at 37°C, and this self-aggregation was monitored by the absorbance at 405 nm (Figure 5). Fibril formation was completely inhibited by 3 μg/ml recombinant decorin. For comparison with the native, or tissue-derived decorin, the activity of the bovine tendon decorin, which had been isolated under denaturing conditions (4 M Gdn HCl), was measured. The native decorin contains a single GAG chain and three N-linked oligosaccharides. As shown in Figure 5, the activity of this sample was less than that of the recombinant decorin. The difference in activity might be due to the exposure of tissue-derived decorin to denaturants, as suggested by Svensson *et al.* (17). Therefore, we then exposed the recombinant decorin to 4 M Gdn HCl and found that the level of Gdn HCl-treated recombinant decorin activity was similar to that of the tissue-derived decorin. This result indicated that the functional conformation of decorin which has been treated with Gdn HCl is not completely restored and that the GAG chain is not involved in the inhibition of fibrillogenesis.

DISCUSSION

Overproduction of TGF-β is thought to be a major cause of tissue fibrosis affecting kidney, liver, lung and skin (23). Decorin binds TGF-β and modulates its activity. This function as well as its inhibitory activity against collagen fibrillogenesis has made decorin an attractive potential therapeutic agent against fibrotic disorders such as glomerulonephritis (13,14). To realize clinical application and to delineate the molecular interactions more precisely, the production of biologically active recombinant decorin is urgently needed. To date, recombinant decorin has been generated in *E. coli* (24-26) and in vaccinia virus-infected mammalian cells (27). The major obstacle encountered in using the *E. coli* system was insolubility of the expressed decorin. In one study, the affinity tag of polyhistidine added to the construct improved the solubility of the fusion protein (24). Alternatively, the protein aggregate forming bacterial inclusion bodies in *E. coli* was solubilized with Gdn HCl before purification of recombinant decorin (26). In the vaccinia virus system, HT1080 cells secreted the N-glycosylated decorin in a soluble form into the medium. Approximately 75 % of the recombinant decorin carried GAG chains. In the present study, we employed a widely used baculovirus system to produce post-translationally modified decorin. The entire cDNA encoding preprodecorin was introduced into the baculovirus. The translated preprodecorin was processed normally in Sf21 cells, and a large quantity of mature decorin was secreted into the medium. The secreted decorin was devoid of GAG chains, suggesting a small or absent capacity to synthesize GAG in the baculovirus-infected insect cells. Mann *et al.* previously reported that decorin mutated at the GAG attachment site and lacking GAG chains was secreted by COS-1 cells (28). Consistent with their results, we also found that modification with GAG chains is not essential for decorin secretion from eukaryotic cells. In contrast, the secreted decorin was N-glycosylated and this glycosylation was apparently required for the secretion of decorin by Sf21 cells, considering that the unmodified core protein was detected in cell lysates but not in the conditioned medium.

A novel strategy for purifying the secreted recombinant decorin was developed in the present study. Baculovirus-infected insect cells synthesize predominantly the high-mannose type N-glycans, while the complex type and truncated chains are also produced later by the N-glycan processing which is dependent upon post-infection periods (29). Nevertheless, most of the resulting oligosaccharides have affinity for Con A. These characteristics of the glycosylation machinery of insect cells prompted us to use Con A-affinity chromatography as a purification step in deriving the N-glycosylated recombinant decorin expressed in Sf21 cells. Simple purification was also achieved by efficient removal of other proteins by applying the preceding hydroxyapatite chromatography.

Several studies have indicated that molecular interactions with ECM proteins occur via the core structure rather than the GAG moiety of decorin (8,11,12), and that the anti-adhesion and anti-fibrotic activities of decorin presumably depend upon proper folding of the core protein (17). In fact, in spite of GAG deletion, the Sf21 cell-derived recombinant decorin was active in

terms of the inhibition of collagen fibrillogenesis. The recombinant decorin purified under non-denaturing conditions displayed remarkable activity, but the activity of decorin which had been treated with Gdn HCl was impaired. Although Hering *et al.* recently reported that Gdn HCl-treated decorin is properly refolded by dilution of the chaotrope so as to achieve a minimally denaturing condition and disulfide shuffling (26), our results has clearly demonstrated that a large quantity of biologically active decorin can be obtained in a baculovirus system without further refolding procedures. The purified decorin will be a potentially useful probe for investigating the molecular interactions of this protein with other ECM proteins and may also have therapeutic applications.

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