ABSENCE OF PROTEOGLYCAN CORE PROTEIN IN THE CARTILAGE MUTANT NANOMELIA

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1. Introduction

The chondrocytes of the homozygous recessive avian mutant nanomelia synthesize sulfated glycosaminoglycans at levels which are 10% of normal. This deficiency results from a reduction in the synthesis of the major cartilage proteoglycan species (PGS-I) to levels 1–2% of normal. The mutation does not affect the synthesis of either the minor proteoglycan of cartilage (PGS-II) [1] or the synthesis of proteoglycans in non-cartilaginous tissues (skin fibroblasts [2] and limb mesenchyme [3,4]). The small amount of PGS-I which is synthesized by nanomelic chondrocytes is of the cartilage type based on its sedimentation rate on sucrose gradients and immunological cross-reactivity [5].

The abnormally low quantities of PGS-I synthesized by the mutant could result from reduced amounts of the core protein of this proteoglycan [4]. To examine directly the nature of the nanomelic defect, intracellular proteins synthesized by both normal and mutant sternal chondrocytes were analyzed.

2. Materials and methods

2.1. *Cell culture*

Sterna were removed from 14-day chick embryos, dissected free of perichondrium, and incubated in a 0.1% pronase (Calbiochem), 0.2% collagenase (Worthington) solution [6] for 1 h at 37°C with gentle stirring. Single cell suspensions were obtained by forcing the digest through two layers of Nitex monofilament screen. The cells were pelleted, rinsed, and resuspended in Ham's F-12 (Gibco) medium supplemented with 10% fetal bovine serum (Sterile Systems), 1 mg streptomycin/ml, 50 U penicillin/ml and 2 μg fungizone/ml. Cells were plated in 4 ml medium at 1×10^6 cells/60 mm bacterial culture dish (Falcon). The suspension cultures were maintained at 37°C in a 5% CO_2, 95% air atmosphere for 72 h. Cell cultures were pulse-labeled for 15 min with 300 μCi/plate of L-[^{35}S]cysteine (New England Nuclear, spec. act. >500 Ci/mmol). Following the incubation in the presence of the radiolabeled amino acid, the chondrocytes were pelleted at 10 000 × *g* in a Beckman microfuge for 1–2 min, and the pellet was solubilized in hot (70°C) electrophoresis sample buffer (100 mM dithiothreitol, 4% SDS, 0.16 M Tris–HCl (pH 6.8), 20% glycerol) and thoroughly stirred using a Vortex mixer.

2.2. *Immunoprecipitation of cell extracts*

Proteins were immunoprecipitated from cell extracts utilizing a double antibody technique. Aliquots of SDS extracts (routinely 50 μl) were diluted 1:1 with buffer (150 mM NaCl, 50 mM Tris–HCl (pH 7.4), 0.05% Nonidet-P40) containing protease inhibitors [7] (50 mM EDTA, 100 mM 6-aminocaproic acid, 5 mM benzamidine–HCl) and dialyzed thoroughly against the same buffer for 2 days at 4°C. Following dialysis, the volume of extract was adjusted to 160 μl and 20 μl rabbit anti-avian proteoglycan monomer serum (anti-A_1D_1 1400 V_o) [8] or normal rabbit serum were added and incubated overnight at 4°C. An amount (120 μl) of sheep anti-rabbit IgG (F_c fragment-specific, Cappel Labs.), needed to maximally precipitate the first antibody, was added and the mixture then incubated for 2 days at 4°C. Immunoprecipitates were pelleted at 10 000 × *g* for

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2 min in a Beckman microfuge. Pellets were dissolved in electrophoresis sample buffer (50 μl) and Vortex mixed.

2.3. *Polyacrylamide gel electrophoresis*

Samples were heated in a 100°C water bath for 5 min prior to electrophoresis. ^{35}S-labeled proteins were resolved in a 0.1% SDS discontinuous slab gel [9] with a 3.12% acrylamide stacking gel (0.08% bis-acrylamide, 125 mM Tris–HCl, pH 6.8) and a 4–15% acrylamide gradient separator (0.29–0.86% bis-acrylamide, 375 mM Tris–HCl, pH 8.8). Samples were electrophoresed through the gel at 40 mA for 4.3 h with a 5.0 mM Tris–HCl, 384 mM glycine, and 0.5% SDS running buffer. Following electrophoresis, slab gels were fixed and stained in 10% (v/v) acetic acid, 25% (v/v) methanol, and fluorographed according to [10], with the exception that ENHANCE (New England Nuclear) was substituted for DMSO-PPO.

3. Results

Radiolabeled extracts of suspension cultured chondrocytes from sterna of 14-day embryonic normal and nanomelic chicks were electrophoresed into denaturing acrylamide-gradient slab gels and fluorograms of the intracellular proteins of $M_r > 46\,000$ were examined. The electrophoretic pattern of radiolabeled proteins from normal chondrocytes contains a band which is absent in the extract of the mutant (fig.1). No other differences were observed between the electrophoretic patterns of the extracts of both genotypes. Based on the migration of globular protein M_r standards, app. M_r $(2.46 \pm 0.21) \times 10^5$ was assigned to the band seen in normal extracts (fig.2). The relationship of the M_r 246 000 band to the proteoglycan PGS-I was established by indirect antibody immunoprecipitation. Utilizing an antiserum elicited

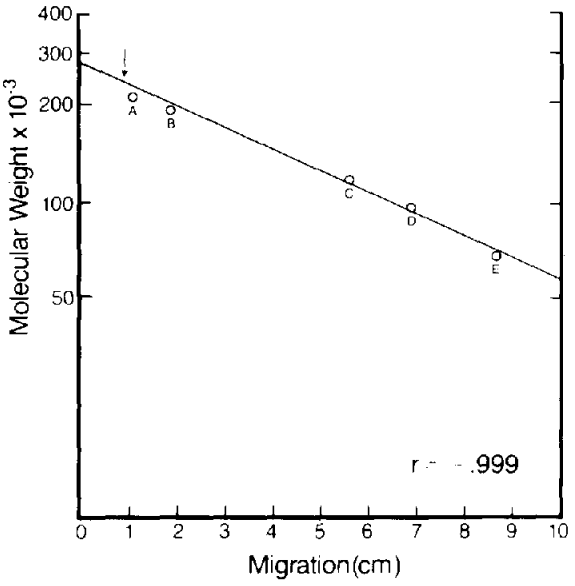

Fig.1. Fluorogram of [^{35}S]cysteine labeled proteins from (1) normal and (2) nanomelic chondrocytes. Chondrocytes of both genotypes were pulse labeled from 15 min and their proteins were resolved in a 4–15% acrylamide gradient SDS slab gel. Arrow indicates a band present in the extract of normal that is absent in the extract of mutant chondrocytes.

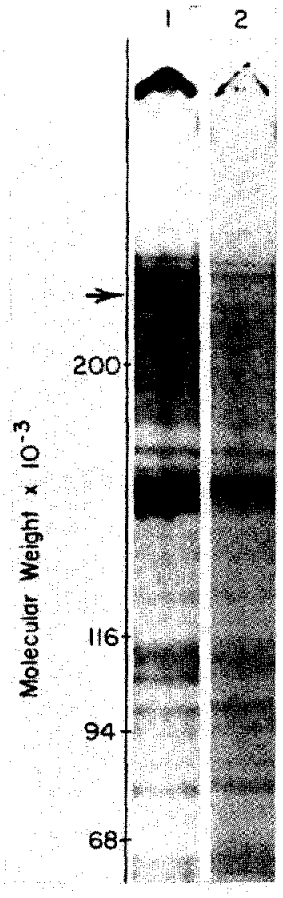

Fig.2. Plot of relative molecular mass (molecular weight) *vs* migration distance of globular protein molecular mass standards electrophoresed in 4–15% acrylamide gradient SDS gels. The molecular mass markers used with their M_r-values were: (A) filamin, 240 000; (B) myosin, 200 000; (C) β-galactosidase, 116 500, (D) phosphorylase B, 94 000; (E) bovine serum albumin, 68 000. Arrow indicates migration distance of band designated in lane 1, fig.1.

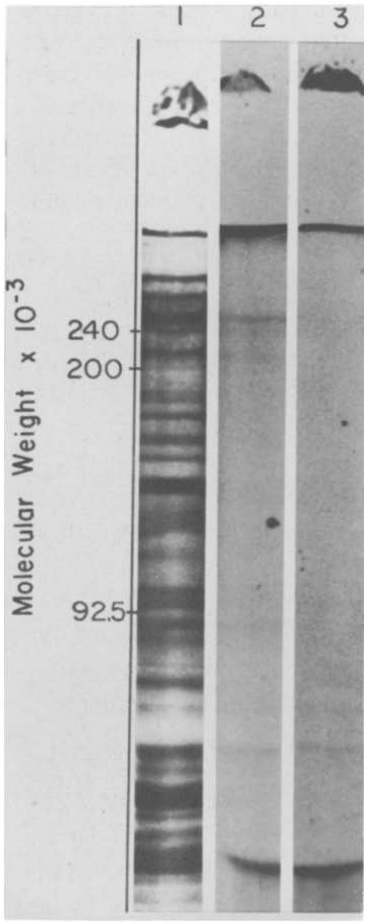

Fig.3. Indirect antibody immunoprecipitation of radiolabeled intracellular proteins of normal chondrocytes utilizing antiserum elicited against cartilage proteoglycan monomer (A_1D_1 CPG-1400 V_o): (1) radiolabeled proteins from normal chondrocytes; (2) immunoprecipitate obtained utilizing anti-cartilage proteoglycan monomer serum; (3) immunoprecipitate obtained with normal rabbit serum. This extract was derived from a separate cell preparation than that used for fig.1. The fluorogram of lanes 2 and 3 were slightly over-exposed to visualize precipitin bands.

against juvenile cartilage proteoglycan monomer, the M_r 246 000 band could be specifically precipitated from extracts of normal chondrocytes (fig.3). Nanomelic chondrocyte extracts contained no detectable precipitable material even after prolonged exposure of the fluorogram (not shown).

4. Discussion

The electrophoretic pattern of intracellular proteins of cultured chondrocytes from normal embryonic chick sterna contains a M_r 246 000 band which is not apparent in extracts from nanomelic chondrocytes. The band seen in normal is specifically immunoprecipitable by anti-cartilage proteoglycan serum. The interpretation of these results, together with studies [1–5,11] with the nanomelic mutant, is that the M_r 246 000 protein represents core protein of PGS-I of cartilage.

The M_r of cartilage proteoglycan core protein has been estimated by biophysical methods to be ~200 000 [12,13]. The apparent M_r of the protein band in this study (246 000) is very close to this estimate. However, cell-free synthesis of core protein indicates a primary translation product of M_r 340 000 [14]. The difference between these two M_r-values would imply post-translational processing from a large core protein precursor to a lower M_r species. Although no information is available at present about the state of glycosylation of the M_r 246 000 band, the latter may represent a partially glycosylated intermediate in the biosynthesis of cartilage proteoglycan.

The nanomelic mutation affects proteoglycans of cartilage only and this defect has been shown to be the result of a deficiency of proteoglycan core protein in that tissue. The synthesis of proteoglycans in non-cartilaginous tissues such as skin fibroblasts and limb mesenchymal cells is not affected by the mutation. The indication is that these non-cartilaginous proteoglycans synthesized by the mutant have normal core proteins. This implies that tissue-specific proteoglycans possess different core proteins encoded by separate genes.

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