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Nanomelic chondrocytes synthesize, but fail to translocate, a truncated aggrecan precursor

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SUMMARY

Cartilage extracellular matrix (ECM) is composed primarily of type II collagen and large, link stabilized aggregates of hyaluronic acid and chondroitin sulfate proteoglycan (aggrecan). Maturation and function of these complex macromolecules are dependent upon sequential processing events which occur during their movements through specific subcellular compartments in the constituitive secretory pathway. Failure to complete these events successfully results in assembly of a defective ECM and may produce skeletal abnormalities. Nanomelia is a lethal genetic mutation of chickens characterized by shortened and malformed limbs. Previous biochemical studies have shown that cultured nanomelic chondrocytes synthesize a truncated aggrecan core protein precursor that disappears with time; however, the protein does not appear to be processed by the Golgi or secreted. The present study investigates the intracellular trafficking of the defective aggrecan precursor using immunofluorescence, immunoelectron microscopy and several inhibitors. Results indicate that nanomelic chondrocytes assemble an ECM that contains type II collagen, but lacks aggrecan. Instead, aggrecan precursor was localized intracellularly, within small cytoplasmic structures corresponding to extensions of the endoplasmic reticulum (ER). At no time were precursor molecules observed in the Golgi. In contrast, normal and nanomelic chondrocytes exhibited no difference in the intracellular or extracellular distribution of type II procollagen. Therefore, retention of the aggrecan precursor appears to be selective. Incubation of chondrocytes at 15˚C resulted in the retention and accumulation of product in the ER. After a return to 37˚C, translocation of the product to the Golgi was observed for normal, but not for nanomelic, chondrocytes, although the precursors disappeared with time. Ammonium chloride, an inhibitor of lysosomal function, had no effect on protein loss, suggesting that the precursor was removed by a non-lysosomal mechanism, possibly by ER-associated degradation. Based on these studies, we suggest that nanomelic chondrocytes are a useful model for examining cellular trafficking and sorting events and the processes by which abnormal products are targeted for retention or degradation. Further investigations should provide insight into the mechanisms underlying chondrodystrophies and other related diseases.

Key words: cartilage, endoplasmic reticulum, proteoglycans, intracellular trafficking, mutants

INTRODUCTION

The normal biological function of proteins and protein complexes depends upon the correct sequence of assembly events established during biosynthesis, processing, and transit through specific subcellular compartments. If the required modifications and associated assembly events fail to occur, proteins will not progress through the biosynthetic pathway (Hurtley and Helenius, 1989; Rose and Doms, 1988). Current research is rapidly advancing our understanding of intracellular trafficking and its regulation, and leads to speculation that certain diseases reflect errors in these processes. For example, forms of familial hypercholesterolemia (Pathak et al., 1988), lysosome storage diseases (Lau and Neufeld 1989) and cystic fibrosis (Cheng et al., 1990) may involve defects of synthesis and intracellular trafficking (recently reviewed by Amara et al., 1992).

Chondrodystrophies are genetic diseases often characterized by abnormalities of the cartilage extracellular matrix (ECM) (Goetinck, 1983; Stanescu et al., 1984). As a consequence, skeletal abnormalities develop, such as malformed, shortened limbs and lax, unstable joints which are

predisposed to severe osteoarthritis. A subset of these diseases, including the lethal chick genetic mutant, nanomelia (Landauer, 1965; Mathews, 1967; Palmoski and Goetinck, 1972; Pennypacker and Goetinck, 1976), and the cartilage matrix-deficient mouse (Kimata et al., 1981; Rittenhouse et al., 1978), has a greatly reduced proteoglycan (aggrecan) content in the ECM, leading to a dramatic reduction in the volume occupied by the cartilage ECM. An abnormality of proteoglycan synthesis or metabolism has also been implicated in human pseudoachondroplasia (Stanescu et al., 1984). Normal cartilage ECM is composed primarily of large proteoglycan aggregates which are restrained within a latticework of type II collagen-containing fibers (Hardingham and Fosang, 1992; Wight et al., 1991). Aggrecan, the large aggregating proteoglycan of cartilage, serves to concentrate negative charges in the ECM, thereby providing the high degree of hydration necessary to maintain an expanded cartilage ECM volume. This function reflects the covalent addition of several hundred chondroitin sulfate and keratan sulfate glycosaminoglycan chains to the aggrecan core protein. The 340 kDa core protein, which represents only 10% of the biosynthetically complete monomer, is also modified by the covalent attachment of N- and O-linked oligosaccharides. Aggrecan, link protein (a separate gene product) and hyaluronic acid are assembled extracellularly into large aggregates, which are further associated with the type II collagen-containing fibers. Presecretion assembly events are involved, but are not yet fully characterized. When the complexities of synthesis, processing and assembly of aggrecan and the functional cartilage ECM are considered, a variety of potential defects are easily imagined, and have been implicated in certain chondrodystrophies.

Nanomelia, a lethal autosomal recessive genetic lesion in chickens, has been attributed to reduced aggrecan content in the cartilage ECM (Palmoski and Goetinck, 1972; Pennypacker and Goetinck, 1976), suggesting that nanomelic chondrocytes could provide a model for the study of abnormal intracellular protein processing and trafficking events. Previous biochemical studies of nanomelic chondrocytes have shown that a truncated core protein is synthesized, but does not appear to undergo Golgi processing events such as the acquisition of endoglycosidase H resistance or conversion to a more highly processed intermediate (O'Donnell et al., 1988). In pulse-chase experiments, the aggrecan core protein disappeared with chase time, but was not secreted. These biochemical data suggest that aggrecan precursors may be degraded at a site prior to entry into the Golgi. Identification of this site within the nanomelic chondrocyte may provide information concerning ER-associated protein degradation (Bonifacino and Lippincott-Schwartz, 1991).

The intracellular sites of protein synthesis, processing and sorting events associated with the biosynthesis of aggrecan have been characterized previously in cultured chicken chondrocytes (Campbell and Schwartz, 1988; Pacifici et al., 1984; Vertel and Barkman, 1984; Vertel et al., 1985a,b, 1989; Vertel and Hitti, 1987). Biochemically, chondrocytes in culture, like their *in situ* counterparts, synthesize aggrecan core protein in the ER, process it to its mature form in the Golgi and secrete aggrecan into the medium. Ultrastructurally, cultured chondrocytes exhibit the extensive endoplasmic reticulum and Golgi complex formations typical of chondrocytes in tissue sections. Morphological studies have shown that molecules destined for matrix incorporation follow the typical ER-Golgi secretory pathway, as evidenced by biochemical analysis and by the intracellular immunolocalization of matrix precursors in the ER and Golgi and extracellular localization of molecules in the ECM. In addition, Vertel et al. (1989) have demonstrated the segregation of aggrecan precursors within smooth membrane-bound, tubular-vesicular regions of the ER. This interesting subcompartment may represent a site for processing or assembly events such as xylose addition or the intracellular association of matrix molecules (e.g. aggrecan precursors and link protein), a sorting site for transport of proteins to the Golgi (similar to the transitional ER described in other models), or it may represent an ER-associated degradative compartment.

The present study identifies intracellular sites of accumulation for the aberrant aggrecan core protein within cultured nanomelic chondrocytes using light and electron microscopic immunolocation methods. We establish that although type II collagen is synthesized, secreted and deposited in the ECM of nanomelic chondrocytes as in normal chondrocytes, aggrecan is not. The defective aggrecan precursors are detected in vesicles throughout the cytoplasm, but not in the Golgi. Ultrastructural immunohistochemical analysis demonstrates that the intracellular sites of aberrant precursor accumulation correspond to smooth membrane-bound extensions of the ER. The application of low temperature, used by others to block intracellular transport and ER degradation (Lippincott-Schwartz et al., 1988), results in retention of the aggrecan precursor within the same restricted regions of the ER, while ammonium chloride, known to block lysosomal degradation, has no effect. Thus, nanomelia may represent a naturally occurring model useful for the study of trafficking defects of ECM or other secreted macromolecules, for the study of ER sorting mechanisms, or for the study of ER-associated degradation.

MATERIALS AND METHODS

Materials

Fertile White Leghorn chicken eggs were purchased from Sharp Sales (West Chicago, IL). Fertile nanomelic eggs were provided by The Department of Animal Genetics at the University of Connecticut (Storrs, CT). Trypsin, Ham's F-12 medium, fetal bovine serum, antibiotic-antimycotic mixture, and Hanks' balanced salt solution (HBSS) were obtained from GIBCO (Grand Island, NY). Testicular hyaluronidase was a product of Leo (Helsingborg, Sweden). Goat anti-rabbit IgG, goat anti-guinea pig IgG, and goat anti-rat IgG coupled to fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC) and Fab fragments of goat anti-rabbit IgG coupled to horseradish peroxidase were obtained from Cappel Laboratories (Malvern, PA). Saponin, sodium borohydride, 1,4-diazabicyclo[2.2.2.]octane, diaminobenzidine, ammonium chloride, potassium ferrocyanide and glutaraldehyde were purchased from Sigma Chemical Co. (St. Louis, MO). Osmium tetroxide and hydroxypropyl methacrylate were products of Electron Microscopy Sciences (Port Washington, PA) and tEpon-812 was purchased from Tousimis (Rockville, MD). [^{35}S]Methionine (1150-1350 Ci/mmol), and En-^{3}Hance were pur-

chased from DuPont-New England Nuclear (Boston, MA). Protein A-Sepharose CL-4B was from Pharmacia (Piscataway, NJ).

Cell culture

Cartilage cells were prepared from the sterna of 15-day-old normal White Leghorn chicken embryos and from 15-day-old embryos with the nanomelia genetic trait (Cahn et al., 1967). Nanomelic embryos were selected from crosses of chickens known to be heterozygous for nanomelia, as described previously (O'Donnell et al., 1988). For immunofluorescence studies, cells were cultured as monolayers on gelatinized carbon-coated coverslips at a density of 5×10^5 cells/60 mm tissue culture dish in 3 ml of Ham's F-12 medium containing 10% fetal bovine serum. To study deposition of type II collagen in the ECM, some of the monolayers were cultured in the presence of 50 μM ascorbate, a co-factor necessary for the stabilization of procollagen and collagen fiber formation (Olsen, 1991; Prockop et al., 1979). For electron microscopy, monolayers were grown at the same density on gelatinized 35 mm culture dishes in 1.5 ml of medium. Monolayers were fed fresh medium every other day and several hours before fixation. Cell suspensions, plated at a density of 2×10^6 cells/100 mm Petri plate in 9 ml of medium, were used for biosynthetic labeling experiments. All cultures were incubated at 37˚C in a humidified atmosphere 95% air and 5% CO_2.

For localization of ECM molecules, 4- or 5-day-old chondrocytes on coverslips, cultured in the presence of ascorbate, were rinsed several times with HBSS and fixed in 75% ethanol. Monolayers designated for intracellular localization studies were incubated with testicular hyaluronidase for 10 min at 37˚C to remove extracellular aggrecan (Vertel and Dorfman, 1979) and washed 3 times with HBSS prior to fixation. For low temperature experiments, hyaluronidase-digested cultures were incubated at 15˚C and reversal was accomplished by continued incubation in medium at 37˚C.

Immunofluorescence staining

Fixed cells on coverslips were permeabilized for 2 min with 98% ethanol/ether (1:1, v/v) and air dried (von der Mark et al., 1977). The permeabilized cells were incubated with primary antibodies for 40 min at 37˚C, washed repeatedly with HBSS, followed by incubation for 40 min with goat anti-guinea-pig IgG, goat anti-rat IgG, or goat anti-rabbit IgG coupled to FITC or TRITC. After several HBSS washes, specimens were mounted in glycerol/PBS (9:1, v/v) containing 0.1% 1,4-diazabicyclo[2.2.2.]octane. A Leitz Ortholux microscope with phase-contrast and epifluorescence optics was used to observe and photograph the samples.

Antibodies

Polyclonal rabbit and guinea pig antibodies directed against (1) hyaluronidase-digested aggrecan monomer from embryonic chicken epiphyses, and (2) pepsin extracted type II collagen from adult chicken sterna were prepared (Upholt et al., 1979; Vertel and Dorfman, 1979). Antibody specificity was characterized by radioimmunoassay (Vertel and Dorfman, 1979), immunoprecipitation (O'Donnell et al., 1988; Upholt et al., 1979; Vertel and Hitti, 1987) and immunoblot analysis. The antibodies were shown to immunoprecipitate all intracellular and extracellular forms of aggrecan as well as cell-free translation products of these matrix molecules. Type II collagen antibodies recognize cell-free translated type II (pre)procollagen, intracellular procollagen and extracellular, fibrillar collagen. Although our type II collagen antibodies cannot distingush procollagen from collagen on the basis of immunoreactivity, others have established that the conversion of procollagen to collagen is an extracellular process (Olsen, 1991; Prockop et al., 1979); therefore, we refer to all intracellular forms of collagen as procollagen. The rat monoclonal antibody S103L recognizes an epitope located on the aggrecan core protein as demonstrated by immunoprecipitation of the unmodified aggrecan core protein translated in cell-free reactions (Upholt et al., 1981) and epitope mapping (Dennis et al., 1990; Krueger et al., 1990). S103L and the polyclonal aggrecan antibodies were used interchangeably in the present studies, based on a previous report that established their equivalent reactivity in immunolocalization experiments (Vertel and Barkman, 1984).

Immunoelectron microscopy

This procedure was modified from the procedure described by Brown and Farquhar (1984). After hyaluronidase digestion, chondrocyte monolayers were washed with cold HBSS, fixed in 0.15% glutaraldehyde/HBSS for 10 min at 25˚C and washed repeatedly with HBSS. Fixed cells were treated with 1% sodium borohydride for 30 min to quench unreacted aldehyde groups and restore antigenicity (Eldred et al., 1983). Permeabilization was accomplished by incubation in 0.025% saponin/HBSS (buffer A) for 30 min at room temperature. Cells were incubated in primary antibody (diluted 1:20 in buffer A + 5% goat serum) or normal rabbit serum at 37˚C for 60 min, followed by repeatedly washing in buffer A. Samples were incubated for 60 min at 37˚C in horseradish peroxidase-coupled goat anti-rabbit IgG Fab fragments (diluted 1:75 in buffer A + 5% goat serum) and repeatedly washed in buffer A and in 0.1 M sodium phosphate buffer. Horseradish peroxidase-linked antibody products were visualized by incubation in 0.2% diaminobenzidine/0.1 M sodium phosphate buffer for 15 min following the addition of H_2O_2 (final concentration, 0.01%). After rinses in 0.1 M sodium phosphate buffer and 0.1 M sodium cacodylate (pH 7.4)/7.5% sucrose (buffer B), the samples were fixed for 60 min in 1.5% glutaraldehyde/buffer B. Subsequently, the cells were washed in buffer B and post-fixed in 1% OsO_4/1.5% $K_4Fe(CN)_6$ in buffer B. Dehydration through a series of ethanol solutions, and through a graded series of hydroxypropyl methacrylate and tEpon solutions, and embedment in tEpon were accomplished according to the method described by Brinkley et al. (1967). Sections were cut parallel to the plane of the monolayers and observed in a Zeiss EM10C transmission electron microscope.

Biosynthetic labeling

Chondrocytes from 4-day-old control and nanomelic suspension cultures were labeled as described by O'Donnell et al. (1988). Control cells were hyaluronidase digested for 10 min at 37˚C prior to HBSS/10% F-12 wash and resuspension. Cells were pulse labeled at 37˚C for 5 min with 75-100 μCi [^{35}S]methionine in HBSS/10% F-12 and chased in 4 ml of medium containing an excess of unlabeled methioine. For low temperature samples, gas-equilibrated medium cooled to 15˚C was added for the chase and the cells were incubated at 15˚C. For reversal, cells were returned to 37˚C. In NH_4Cl experiments, suspension cultures were pre-incubated with 50 mM NH_4Cl for 30 min prior to labeling, and pulse labeled and chased in the presence of NH_4Cl at the same concentration. Aliquots were taken for biochemical analysis at the indicated times, centrifuged in a microfuge, washed with cold HBSS by repeated suspension and centrifugation, and treated for immunoprecipitation or directly for gel electrophoresis.

Immunoprecipitations

Immunoprecipitations were performed as a modification of the procedure of Bumol and Reisfeld (1982), as described by O'Donnell et al. (1988). Briefly, radiolabeled cells were solubilized in 2% SDS, HBSS by boiling for 2 min, quickly cooled on ice, and diluted with immunoprecipitation buffer (50 mM Tris, pH 7.4/190 mM NaCl/6 mM EDTA/2.5% Triton X-100). Samples were reacted with the S103L antibody pre-adsorbed to Protein A-Sepharose beads overnight with shaking in the cold, washed exten-

sively with buffer (phosphate-buffered saline/0.5% Triton-X 100/0.1% ovalbumin) and prepared for gel electrophoresis as described below.

SDS-polyacrylamide gel electrophoresis

Labeled cell samples and immunoprecipitates were solubilized in gel sample buffer (75 mM Tris-HCl (pH 6.7)/2% SDS/20% glycerol/0.003% bromophenol blue/0.1 M dithiothreitol) by heating at 70˚C for 30 min and electrophoresed on 3% to 5% gradient polyacrylamide slab gels containing 0.1% SDS (Neville, 1971; Vertel and Hitti, 1987). Gels were treated with En-^{3}Hance according to the directions of the manufacturer and dried for autoradiographic fluorography (Laskey and Mills, 1975).

RESULTS

Nanomelic chondrocytes do not accumulate extracellular aggrecan

When chondrocytes from normal chicken embryos are grown in culture in the presence of 50 μM ascorbate, they synthesize, secrete and deposit a cell-associated ECM. Based on immunofluorescent staining, this ECM contains a fibrillar network of type II collagen (Fig. 1b) and aggrecan (Fig. 2b). The large spaces between chondrocytes which are observed in the accompanying phase-contrast micrographs (Figs 1a, 2a) reflect the contribution of aggrecan to the

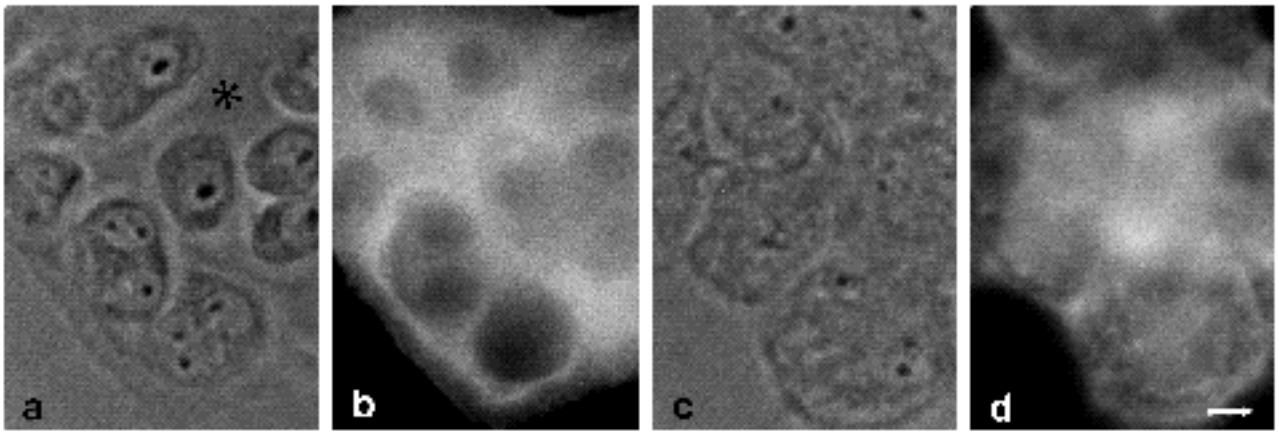

Fig. 1. Both normal and nanomelic chondrocytes deposit type II collagen into the ECM. Normal (a,b) and nanomelic (c,d) chondrocytes were grown in monolayer culture with ascorbic acid supplements to maximize matrix deposition. At 5 days of culture, cells were fixed with ethanol, prepared for immunofluorescence localization and reacted with antisera for type II collagen, as described in Materials and Methods. Phase-contrast micrographs of immunostained cells and the associated ECM contrast the matrix-filled spaces (asterisk) between normal chondrocytes (a) with the close apposition of nanomelic chondrocytes (c). Deposition of type II collagen into the cell-associated ECM is observed for normal (b) and nanomelic (d) chondrocytes.

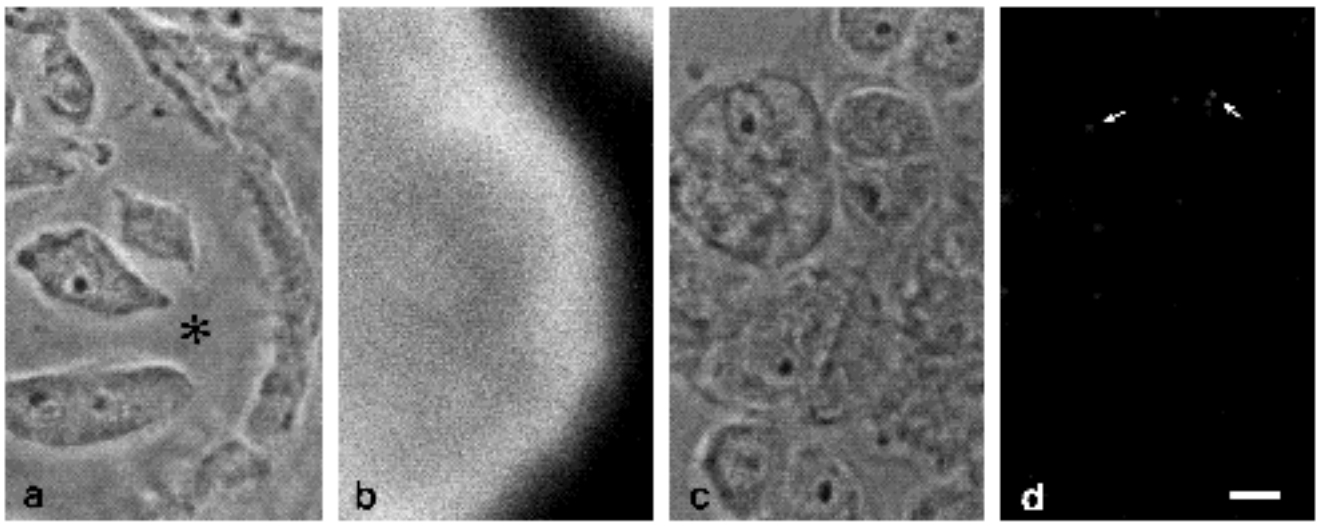

Fig. 2. Normal, but not nanomelic, chondrocytes deposit aggrecan into the ECM. Chondrocytes grown with ascorbic acid supplements were fixed at 5 days of culture, prepared for immunofluorescence localization, and reacted with the S103L monoclonal antibody, as described in Materials and Methods. Phase-contrast micrographs of immunostained cells and the associated ECM again show the large, matrix-filled spaces (asterisk) that separate normal chondrocytes (a) and the close proximity of nanomelic chondrocytes (c). Normal chondrocytes deposit abundant amounts of aggrecan into the cell-associated ECM (b), while nanomelic chondrocytes do not (d). The positive immunoreactivity of a few intracellular vesicles in nanomelic chondrocytes (arrows) suggests that the aberrant nanomelic product does not leave the cell.

maintenance of a net negative charge, which serves to increase the level of hydration and thereby increase the volume occupied by the ECM.

Chondrocytes from nanomelic chicken embryos cultured under similar conditions also accumulate an ECM. As shown in Fig. 1d, fibrillar type II collagen is deposited extracellularly. In contrast to the cells in normal chondrocyte cultures, nanomelic chondrocytes are closely associated with each other, and are not separated by large, matrix-filled spaces (Figs 1c, 2c). Immunofluorescent localization of aggrecan antibodies reveals the lack of ECM reactivity (Fig. 2d). Instead, small cytoplasmic vesicles exhibit immunoreactivity (arrows, Fig. 2d).

Nanomelic chondrocytes synthesize and secrete type II procollagen, but synthesize and do not secrete aggrecan precursors

The observation of intracellular precursors of ECM molecules is obscured by the extensive ECM which accumulates around normal chondrocytes (e.g. Figs 1b, 2b). However, if cells are cultured in the absence of ascorbate supplements, type II procollagen is synthesized and secreted, but is not deposited extracellularly (ascorbate is a cofactor required for hydroxylation of proline and subsequent triple helix formation and fibril assembly (Olsen, 1991; Prockop et al., 1979)). Under these conditions, digestion with hyaluronidase prior to fixation will effectively remove extracellular aggrecan. As a result, intracellular precursors are readily observed after immunofluorescent staining. As shown, type II procollagen is localized throughout the cytoplasm in both normal (Fig. 3a) and nanomelic (Fig. 3b) chondrocytes. The pattern of immunostaining between the two is indistinguishable, indicating that the synthesis and processing of type II procollagen are the same for both normal and mutant cells.

In contrast, the pattern of intracellular immunofluorescence differs markedly for aggrecan precursors in normal and nanomelic chondrocytes. In normal chondrocytes (Fig. 4a), aggrecan precursors are observed in the perinuclear region and in additional cytoplasmic vesicles. Previous studies have demonstrated that the perinuclear staining corresponds to the Golgi and that the other cytoplasmic vesicles represent segregated regions of the ER (Vertel and Barkman, 1984; Vertel et al., 1985a,b, 1989). In nanomelic chondrocytes, immunofluorescence localization is restricted to vesicles scattered throughout the cytoplasm (Fig. 4b). No staining of the perinuclear Golgi is observed.

In order to determine the intracellular sites of aggrecan precursor accumulation in nanomelic chondrocytes, ultrastructural studies using immunoperoxidase localization were undertaken. As shown in Fig. 5, immunoreactive product is observed only in membrane-bound regions continuous with the rough ER. Vesicles in the Golgi area are devoid of immunostaining.

Low temperature inhibits the loss of the mutant aggrecan precursor while ammonium chloride does not

In previously reported experiments (O'Donnell et al., 1988), we identified a 300 kDa nanomelic glycoprotein related to the 370 kDa aggrecan core protein precursor. Pulse-chase labeling studies demonstrated a time-dependent loss of the truncated precursor and the absence of modified biosynthetic intermediates, suggesting that the 300 kDa nanomelic

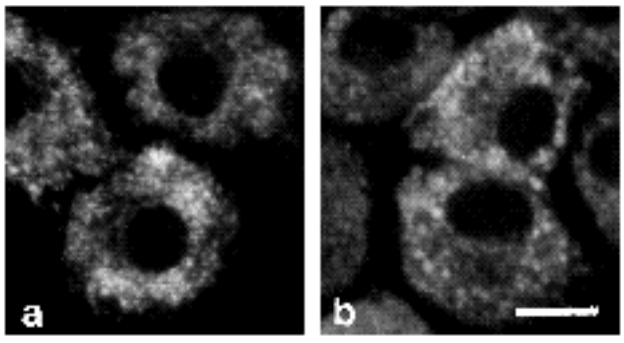

Fig. 3. Comparable intracellular compartments are involved in the synthesis and processing of type II procollagen in normal and nanomelic chondrocytes. The immunofluorescent localization of type II collagen antisera is shown within normal (a) and nanomelic (b) chondrocytes grown in culture for 5 days without ascorbic acid supplements. Note that the intracellular distribution of type II procollagen is the same for normal and nanomelic chondrocytes. In each case, type II procollagen is observed in cytoplasmic structures that have been shown in previous light and electron microscopic studies to correspond to the rough ER (Vertel et al., 1985a,b, 1989).

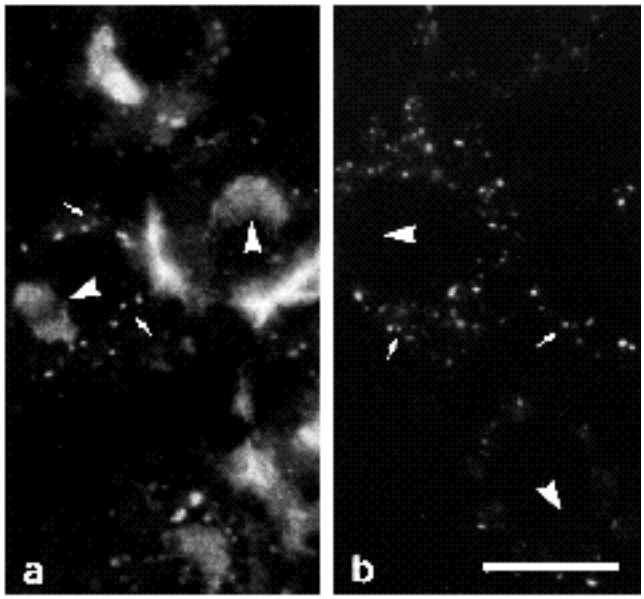

Fig. 4. The intracellular distribution of aberrant aggrecan precursors is abnormal in nanomelic chondrocytes. Extracellular aggrecan was removed by hyaluronidase digestion prior to fixation. In normal chondrocytes (a), immunostaining of intracellular aggrecan precursors is evident in the region of the perinuclear Golgi (arrowheads) and in other vesicles of the cytoplasm (small arrows). In nanomelic chondrocytes (b), reactivity is observed only in vesicles of the cytoplasm (small arrows), and is excluded from the region of the perinuclear Golgi (arrowheads).

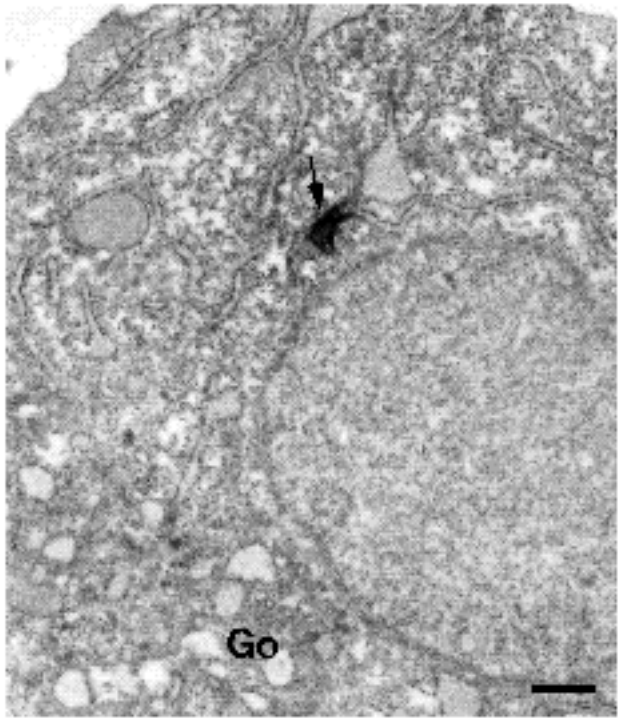

Fig. 5. Abnormal aggrecan precursors in nanomelic chondrocytes are accumulated in subcompartments of the ER. Ultrastructural studies of immunoperoxidase-localized aggrecan core protein antibodies in nanomelic chondrocytes demonstrate immunoreactivity within membrane-bound regions continuous with ribosome-studded regions of the ER (arrow). Vesicles in the area of the Golgi are devoid of immunostaining (Go).

glycoprotein was degraded intracellularly and not processed further. Since incubation at low temperature will inhibit intracellular degradation (Lippincott-Schwartz et al., 1988), we investigated the effects of low temperature on the synthesis, processing and loss of the nanomelic glycoprotein. As shown in Fig. 6, the 300 kDa nanomelic glycoprotein synthesized in the initial pulse disappears as a function of chase time at 37˚C (Fig. 6A), but remains throughout the chase when cells are incubated at 15˚C (Fig. 6B). However, when the temperature is returned to 37˚C, the product is rapidly lost (Fig. 6C). Although an increase in immunofluorescence staining for aggrecan precursor is observed in nanomelic chondrocytes after incubation at low temperature, the product remains distributed throughout the cytoplasm (Fig. 7c). When the temperature is shifted back to 37˚C, immunofluorescence staining continues to be localized within the cytoplasm, and fails to be observed in the region of the perinuclear Golgi (Fig. 7d). In contrast, aggrecan precursor in normal chondrocytes accumulates in the cytoplasm at low temperature (Fig. 7a), but is translocated rapidly to the Golgi when the incubation temperature is returned to 37˚C (Fig. 7b). Immunoelectron microscopic analysis demonstrated that the subcellular compartments containing accumulated product were smooth membrane-limited extensions of the ER similar to those shown in Fig. 5 for nanomelic chondrocytes incubated at 37˚C.

In order to determine whether lysosomes play a role in the time-dependent loss of the aberrant nanomelic precursor, we tested the effect of NH_4Cl, an agent known to inhibit lysosomal degradation (Lippincott-Schwartz et al., 1988). No difference in the rate or extent of product loss was observed between nanomelic chondrocytes treated with 50 mM NH_4Cl (Fig. 8B) and controls (Fig. 8A), suggesting that lysosomes are not involved in this loss. As expected, intracellular immunostaining with aggrecan antibodies remained cytoplasmic in nanomelic chondrocytes after NH_4Cl treatment (not shown).

DISCUSSION

The lethal genetic defect nanomelia is characterized by shortened, malformed limbs and other skeletal abnormalities which can be attributed to deficiencies in aggrecan, the large aggregating proteoglycan of cartilage (Palmoski and Goetinck, 1972; Pennypacker and Goetinck, 1976). In a previous biochemical study of nanomelic chondrocytes, O'Donnell et al. (1988) identified a 300 kDa glycoprotein related to the normal 370 kDa aggrecan core protein precursor. In normal chondrocytes, conversion of the 370 kDa core protein precursor to aggrecan involves extensive, time-dependent, post-translational modifications. The 300 kDa product of nanomelic chondrocytes exhibited endoglycosidase H-sensitivity and in contrast, after pulse labeling, disappeared with increasing time of chase. The study concluded that the truncated nanomelic precursor did not undergo further post-translational modification and suggested that it was degraded.

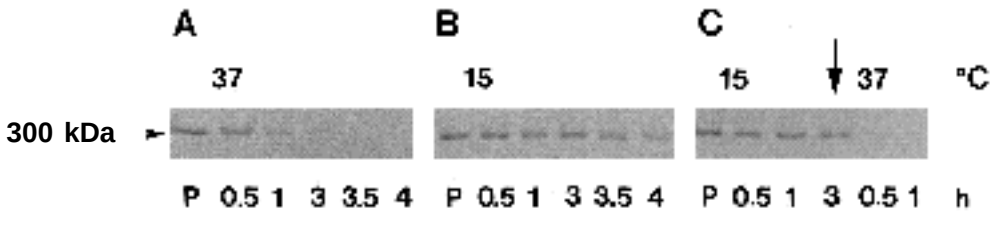

Fig. 6. Incubation at low temperature reversibly inhibits the time-dependent loss of the truncated aggrecan precursor in nanomelic chondrocytes. Nanomelic chondrocytes grown in suspension culture for 4 days were pulse labeled for 5 min with [^{35}S]methionine at 37˚C (P) and chased in complete medium at 37˚C (A) or 15˚C (B). In reversal experiments (C), medium at 15˚C was replaced with medium at 37˚C at 3 h of chase (arrow), and incubation was continued at 37˚C. Cells were collected at the indicated times, and immunoprecipitated with the S103L monoclonal antibody, as described in Materials and methods. Note that the truncated aggrecan precursor disappears with time at 37˚C (A), is retained at 15˚C (B), and quickly disappears when the temperature is shifted up to 37˚C (C).

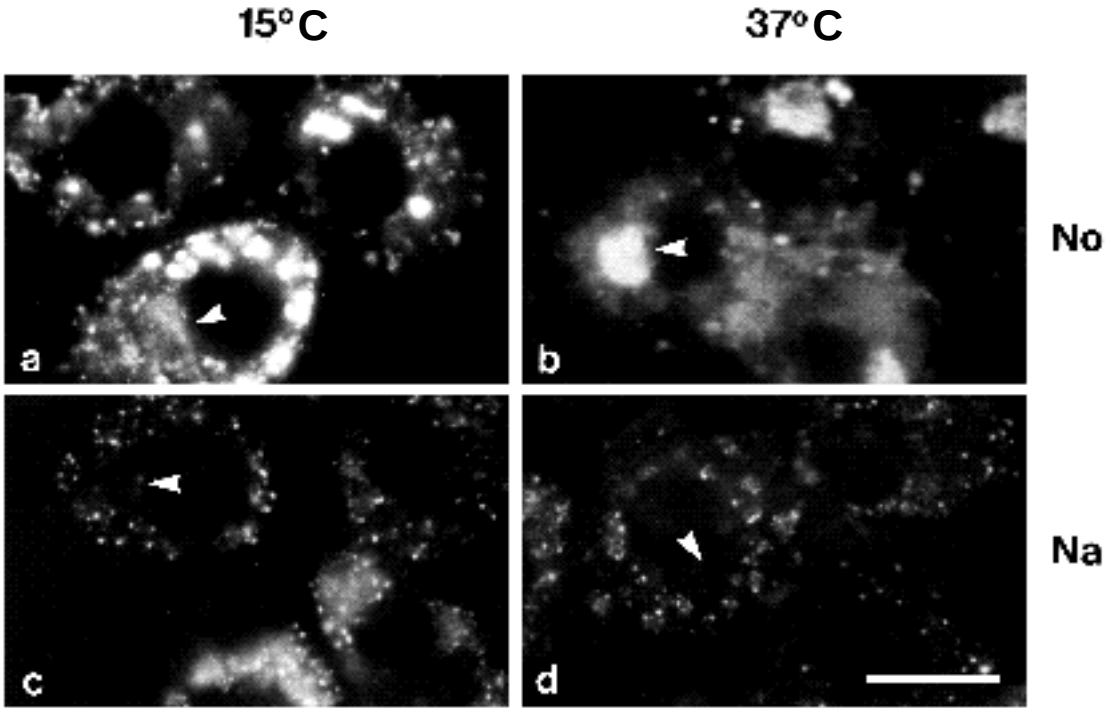

Fig. 7. Aggrecan precursors that accumulate at 15˚C in normal chondrocytes are translocated to the Golgi upon release from the temperature block, but the related product of nanomelic chondrocytes remains in the ER and, with time, returns to steady-state levels. At 4 days of culture, extracellular aggrecan was removed by hyaluronidase digestion. Normal (a) and nanomelic (c) chondrocytes were incubated at 15˚C for up to 4 hours and fixed with ethanol. Reversal from the low temperature block was accomplished by replacement with medium at 37˚C and continued incubation for an additional 20 min at the normal temperature of 37˚C prior to fixation (b,d). Immunostaining for aggrecan precursors demonstrates the accumulation in cytoplasmic ER regions of both normal (a) and nanomelic (c) chondrocytes at low temperature. Upon reversal of the 15˚C block, aggrecan precursors within normal chondrocytes are translocated to the perinuclear Golgi (b). In contrast, accumulated precursors in nanomelic chondrocytes remain in vesicular structures throughout the cytoplasm and are absent from the region of the perinuclear Golgi (d). Arrowheads indicate the region of the perinuclear Golgi.

Data in the present report demonstrate that the truncated aggrecan precursor progresses no further than the ER, and is perhaps degraded there. Immunolocalization studies demonstrate the failure of aggrecan-related product to be detected in the perinuclear Golgi or in the cell-associated ECM of nanomelic chondrocytes. Instead, immunoreactive product was located only in smooth-membrane-bound regions continuous with the rough ER. Our results demonstrate that the time-dependent loss of the aberrant aggrecan precursor in nanomelic chondrocytes is inhibited by incubation at low temperature, but is not changed by treatment with NH_4Cl, an agent known to inhibit lysosomal degradation (Lippincott-Schwartz et al., 1988). The biochemical and morphological data are consistent with the notion that the aberrant 300 kDa nanomelic glycoprotein is not translocated to the Golgi, is retained in a smooth-membrane-bound compartment of the ER, and is subject to degradation there. The defective processing observed for the aberrant aggrecan core protein is apparently selective, since the synthesis, secretion and deposition of type II collagen in the ECM are unaffected in nanomelic chondrocytes.

The aborted trafficking of defective cell products such as the truncated nanomelic glycoprotein has been implicated in several other genetic diseases (Amara et al., 1992). For example, fibroblasts from patients with familial hypercholesterolemia synthesize and glycosylate mutant low density

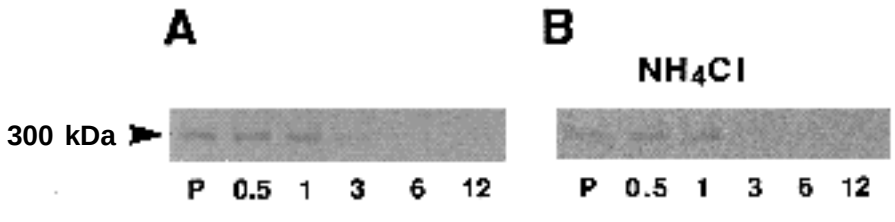

Fig. 8. Time-dependent loss of the truncated aggrecan precursor in nanomelic chondrocytes is unaffected by NH_4Cl. Nanomelic chondrocytes grown in suspension culture for 4 days were pulse labeled for 5 min with [^{35}S]methionine (P) and chased in complete medium with (B) or without 50 mM NH_4Cl (A). Chondrocytes treated with NH_4Cl were preincubated for 30 min and pulse labeled in the presence of the lysomotrophic agent. Cells were collected at the indicated times, and immunoprecipitated with the S103L monoclonal antibody, as described in Materials and methods. Note that the truncated aggrecan precursor disappears with time in the absence (A) or presence (B) of NH_4Cl.

lipoprotein receptors that do not reach the cell surface, but accumulate in the ER (Pathak et al., 1988). Lau and Neufeld (1989) demonstrated that the accumulation of gangliosides in lysosomes of a patient with Tay-Sachs disease resulted from the production of a truncated precursor of the A isoenzyme of lysosomal β-hexosaminidase that was retained and degraded in an early biosynthetic compartment (presumably the ER) rather than being delivered to the lysosome. Cheng et al. (1990) suggested that defects in the synthesis and intracellular trafficking of the cystic fibrosis transmembrane conductance regulator are responsible for most cystic fibrosis. Several types of chondrodystrophies, including achondroplasias and pseudoachondroplasias, are characterized by the intracellular accumulation of ECM precursors that may result from abnormalities in the translocation of these molecules (Stanescu et al., 1984). Knowledge of the relationship between the synthesis of aberrant precursors and problems in their intracellular trafficking is likely to lead to new insights regarding the clinical progression of genetic abnormalities.

Diseases involving defective trafficking reflect a general cellular mechanism that exerts quality control through the detection and removal of abnormal proteins. When necessary conformational and assembly events do not occur, products utilizing the secretory pathway are either retained in the ER or targeted for degradation (Bonifacino and Lippincott-Schwartz, 1991; Hurtley and Helenius, 1989; Rose and Doms, 1988). This mechanism has been particularly well documented for viral glycoproteins (Copeland et al., 1986, 1988; Doms et al., 1987, 1988; Gething et al., 1986) and membrane receptor complexes. Studies of the T cell antigen receptor (a seven subunit oligomer) demonstrate that retention and degradation in the ER are dependent upon the inherent properties of the individual subunits and their assembly into partial or complete multimeric complexes (Bonifacino et al., 1989, 1991; Chen et al., 1988; Suzuki et al., 1991). For example, as single subunits or incompletely assembled complexes, the α and β subunits are subject to rapid ER degradation (Lippincott-Schwartz et al., 1988). In cells deficient in expression of the β subunit, rapid, non-lysosomal degradation of the δ chain was observed, while the ε, γ and ξ subunits were retained, apparently within the ER (Chen et al., 1988). In the case of the H1 subunit of the asialoglycoprotein receptor, coexpression with the H2 subunit leads to the formation of a hetero oligomer that is translocated to the cell surface, but when expressed alone, is degraded in the ER (Amara et al., 1989; Wikstrom and Lodish, 1991). Recent evidence establishes that the initial proteolytic cleavage event occurs in the ER and further suggests that once a protein is targeted for degradation it has no alternative fate. Secreted products such as the immunoglobulins are subject to similar regulatory controls. In the absence of light chain synthesis, both secreted and transmembrane immunoglobulin heavy chains are retained in the ER (Bole et al., 1986).

It is likely that the truncated aggrecan precursor in nanomelic chondrocytes is retained in the ER and degraded there. The following observations suggest ER retention: (1) immunolocalization studies reveal no immunoreactive product in the Golgi or ECM of nanomelic chondrocytes; (2) secretion is not detected by biochemical analysis; (3) the truncated product is sensitive to endoglycosidase H and exhibits no evidence of further post-translational modification; and (4) immunoreactive product is observed only in smooth-membrane-bound regions continuous with the rough ER. Degradation of the truncated aggrecan precursor is suggested by the following lines of evidence. After pulse labeling, the truncated aggrecan precursor disappears with increasing time of chase. This time-dependent loss is inhibited by incubation at low temperature, but is not affected by treatment with ammonium chloride, an agent known to inhibit lysosomal degradation. When the time-dependent loss of the mutant glycoprotein is inhibited by incubation at low temperature, immunoreactive product is observed to accumulate in a smooth-membrane-enclosed subcompartment of the ER. The return to incubation temperatures of 37˚C fails to result in the translocation of accumulated material to the Golgi. In contrast, product accumulated at low temperatures in normal chondrocytes is rapidly translocated to the perinuclear Golgi.

Accumulation of the truncated glycoprotein is observed in smooth-membrane-bound regions of the nanomelic chondrocyte ER, presumably as a result of post-translational events. The biochemical demonstration of both time-dependent loss of the aberrant precursor and the absence of Golgi-mediated processing would suggest that this compartment, or one with which it is associated, functions in protein sorting or degradation. Sorting might result in translocation to the Golgi or to a separate nonlysosomal degradative site. The segregation of an aberrant precursor destined for degradation in a restricted, smooth-membrane-bound region of the ER is novel, particularly since reports of nonlysosomal degradation in other cells suggest that it occurs throughout the ER lumen or within the rough ER. The utilization of tubular-vesicular regions of the smooth-membrane-limited ER may be uniquely characteristic of chondrocytes, however, since even in normal cultured chicken chondrocytes aggrecan precursors have been observed to accumulate in similar regions of the ER (Vertel et al., 1989, 1992). We have speculated that the smooth-membrane-bound subcompartment of the chondrocyte ER fulfills specific functions that might include some aspect of protein processing, such as xylose addition, the presecretion association of matrix molecules (e.g. aggrecan precursors and link protein), sorting prior to translocation to the Golgi or ER-associated degradation. The possibility that this subcompartment serves a degradative function (or sorts products within it to a degradative site) is supported by the observation that the aberrant nanomelic product, targeted for degradation, accumulates there.

The observed ER retention and possible degradation result from an alteration in the biochemical properties of the defective nanomelic precursor and may have implications for the normal trafficking and function of cartilage aggrecan. The data that follow suggest that the nanomelic glycoprotein is lacking the characteristic C-terminal globular domain. We have shown that nanomelic chondrocytes produce a 300 kDa glycoprotein, approximately 20% smaller than the normal 370 kDa core protein (O'Donnell et al., 1988). This truncated product retains the epitope required for reactivity with the monoclonal antibody 1-C-6, which recognizes the N-terminal, hyaluronic acid bind-

ing region of aggrecan (Hejna et al., 1987). The nanomelic product also contains the epitope required for reactivity with the monoclonal antibody S103L. Recently, it has been shown that S103L recognizes a sequence of 11 amino acids located approximately 25% into the protein from the C-terminal end (Dennis et al., 1990; Krueger et al., 1990). Thus, the S103L site is close to the postulated termination site for the truncated nanomelic glycoprotein, and the N- and C-terminal domains of the aberrant nanomelic product can be defined by the hyaluronic acid binding region and S103L epitopes, respectively. If these domains set the limits of the 300 kDa nanomelic glycoprotein, then we can predict the following features. Presumably, the truncated precursor is capable of interacting with hyaluronic acid and with link protein, since the N-terminal, hyaluronic acid-binding epitope, defined by reactivity with the 1-C-6 antibody, remains intact. Most of the sites for *N*-glycosylation are also found in the region close to the N terminus, and therefore should be present. (In this regard, previous data (O'Donnell et al., 1988) demonstrated the addition of *N*-asparagine-linked oligosaccharides to the truncated nanomelic glycoprotein.) It is likely that the regions utilized for glycosaminoglycan chain addition and elongation remain intact, since these sequences are located NH_2-terminal to the S103L domain. Thus, the truncated precursor should be capable of functioning as a substrate for glycosaminoglycan chain formation, though our data indicate that it does not.

The above analysis strongly suggests that the domain located C-terminal to the S103L epitope is absent from the truncated nanomelic glycoprotein and may be necessary for the translocation and complete maturation of the normal aggrecan precursor. Several interesting features of this domain have been described (recently discussed by Hardingham and Fosang, 1992). Rotary-shadowing studies have demonstrated the globular nature of this region (Paulsson et al., 1987; Wiedemann et al., 1984). Other reports have noted homology of the N-terminal part of the globular domain with the hepatic lectin and homology of its C-terminal portion with complement regulatory protein (Doege et al., 1987; Sai et al., 1986). The expressed globular domain exhibits specific, low-affinity interactions with several sugar ligands (Halberg et al., 1988). Based on these observations, it has been suggested that the domain may interact with the carbohydrate ligands of other ECM molecules and may function in the assembly of the ECM. For many proteins, exit from the ER requires specific conformational changes. If conformational changes in the C-terminal domain of aggrecan are important, they may be required for movement out of the ER. Alternatively, this domain may function as a signal for translocation. It is also possible that truncation leads to the exposure of a C-terminal sequence that serves as a signal for retention. A detailed molecular study of the nanomelic gene would help to resolve some of these issues.

In summary, evidence presented in this and a previous report suggests that chondrocytes from nanomelia embryos produce an aberrant proteoglycan precursor that fails to be further processed and secreted, but is instead aborted at the level of the ER. Based on these results, the nanomelic chondrocyte is likely to be a useful model for elucidating one cellular mechanism involved in the disposal of a defective ECM glycoprotein precursor. The hypothesis that a subset of known chondrodystrophies reflects defects of processing or cellular membrane trafficking suggests the basis for a fruitful approach to their analysis from which we can determine both the nature of these genetic diseases and the normal requirements for the synthesis, processing, translocation and assembly of a functional cartilage ECM. In turn, these events have implications for general aspects of biosynthetic processing, organelle function and membrane trafficking.

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