The chondrodystrophy, nanomelia: biosynthesis and processing of the defective aggrecan precursor

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The lethal chicken mutation nanomelia leads to severe skeletal defects because of a deficiency of aggrecan, which is the large aggregating chondroitin sulphate proteoglycan of cartilage. In previous work, we have demonstrated that nanomelic chondrocytes produce a truncated aggrecan precursor that fails to be secreted, and is apparently arrested in the endoplasmic reticulum (ER). In this study, we investigated the biosynthesis and extent of processing of the abnormal aggrecan precursor. The truncated precursor was translated directly in cell-free reactions, indicating that it does not arise post-translationally. Further studies addressed the processing capabilities of the defective precursor. We found that the mutant precursor was modified by N-linked, mannose-rich oligosaccharides and by the addition of xylose, but was not further processed; this is consistent with the conclusion that it moves no further along the secretory pathway than the ER. Using brefeldin A we demonstrated that the defective precursor can function as a substrate for Golgi-mediated glycosaminoglycan chains, but does not do so in the nanomelic chondrocyte because it fails to be translocated to the appropriate membrane compartment. These studies illustrate how combined cell biological/biochemical and molecular investigations may contribute to our understanding of the biological consequences and molecular basis of genetic diseases, particularly those involving errors in large, highly modified molecules such as proteoglycans.

INTRODUCTION

Recent studies highlight how important sequential processing and assembly events and movement through specific compartments of the secretory pathway are for normal protein function (Rose and Doms, 1988; Hurtley and Helenius, 1989; Farquhar, 1991). By extrapolation, diseases may arise as a consequence of errors in these processes. For example, problems of synthesis, assembly and intracellular trafficking are implicated in the basic mechanisms underlying lysosomal storage diseases, familial hypercholesterolaemia, and cystic fibrosis (Amara et al., 1992). In connective-tissue disorders such as osteogenesis imperfecta and chondrodystrophies, defects may involve the abnormal synthesis, processing, translocation and assembly of extracellular matrix (ECM) molecules (Goetinck, 1983; Stanescu et al., 1984; Johnson, 1986; Prockop, 1990; Spranger and Martoux, 1990; Byers et al., 1991). These types of errors might be expected for ECM molecules, as they are mostly large, highly modified and strongly interactive, and are characteristically assembled into extensive macromolecular arrays. Although defects in the collagens have frequently been found (Prockop, 1990; Byers et al., 1991), proteoglycans and other matrix molecules are also candidates for these genetic diseases (Goetinck, 1983; Stanescu et al., 1984; Johnson, 1986; Spranger and Martoux, 1990). Nanomelia is one such genetic mutation that causes shortened limbs, other skeletal abnormalities and death in chicken embryos as a result of the absence of aggrecan in cartilage ECM (Landauer, 1965; Pennypacker and Goetinck, 1976; Vertel et al., 1993b).

Aggrecan is associated with a network of type-II collagen-containing fibrils in normal cartilage ECM where it contributes localized concentrations of negative charges that serve to increase the level of hydration and thereby provide an expanded tissue volume for bone replacement during long-bone development. Without aggrecan, the intercellular, matrix-filled spaces are greatly reduced, the cartilage model fails to expand and consequently, the growth of long bones is seriously impaired. Aggrecan function principally reflects the more than 100 covalently attached chondroitin sulphate (CS) and keratan sulphate (KS) glycosaminoglycan chains that are the result of complex biosynthetic processes involving thousands of co- and post-translational reactions (reviewed recently by Wight et al., 1991; Hardingham and Fosang, 1992). The large (> 200 kDa) aggrecan core protein is only 10% of the molecular mass of the fully processed proteoglycan, which is modified as well by N- and O-linked oligosaccharides. The chondrocyte is characterized by an extensive endoplasmic reticulum (ER) and Golgi, as expected for a cell heavily committed to the production of highly modified ECM molecules in large quantities. The synthetic, processing and assembly events are accomplished in several subcompartments of the secretory pathway that have been described in previous studies (Pacifici et al., 1984; Vertel and Barkman, 1984; Vertel and Hitti, 1987; Campbell and Schwartz, 1988; Vertel et al., 1985a,b, 1989, 1993a; Kearns et al., 1993), although some features of intracellular processing and assembly are not yet well defined. Structural studies of aggrecan from several species (including chicken) have provided the complete coding sequence and a clear definition of several domains (see Figure 6); these include three highly conserved globular domains, G1 and G2 at the N-terminus, and G3 at the C-terminus, and the domains involved in glycosaminoglycan chain substitution for KS and CS (reviewed by Wight et al., 1991; Hardingham and Fosang, 1992; for chicken aggrecan, see Li et al., 1993; Chandrasekaran and Tanzer, 1992).

Abbreviations used: BFA, brefeldin A; CS, chondroitin sulphate; ECM, extracellular matrix; ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KS, keratan sulphate; FCS, fetal-calf serum.

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In this article, we explore the biological consequences of the nanomelia defect. On the basis of previous work that reported the arrest of a truncated aggrecan precursor in the ER of the nanomelic chondrocyte (Vertel et al., 1993b), we have investigated the origin of the truncated product, the extent of its compartment-specific processing and its potential for additional processing in studies that utilize cell-biological, biochemical and molecular techniques. In concomitant studies reported elsewhere, we identify the defect as a premature stop codon (Li et al., 1993).

MATERIALS AND METHODS

Materials

Fertile White Leghorn chicken eggs were purchased from Sharp Sales (West Chicago, IL, U.S.A.). Fertile eggs with the nanomelia trait were supplied by the Department of Animal Genetics, University of Connecticut (Storrs, CT, U.S.A.). Trypsin, Ham's F-12 medium, fetal-calf serum (FCS), antibiotic–antimycotic mixture and Hank's balanced salt solution were obtained from GIBCO–BRL (Grand Island, NY, U.S.A.). En^{3}Hance, [^{35}S]methionine, UDP-[^{14}C]xylose, and GeneScreen Plus membranes were products of DuPont–New England Nuclear (Wilmington, DE, U.S.A.). $H_2{}^{35}SO_4$ was purchased from ICN Biochemicals (Irvine, CA, U.S.A.). Testicular hyaluronidase was a product of Leo (Helsingborg, Sweden) and chondroitinase ABC was from Miles (Elkhart, IN, U.S.A.). Rabbit reticulocyte lysate translation kits were purchased from Promega (Madison, WI, U.S.A.). Brefeldin A (BFA) was a product of Epicentre Technologies (Madison, WI, U.S.A.). Goat anti-(rabbit IgG) coupled to fluorescein isothiocyanate (FITC) was obtained from Organon Teknika (Durham, SC, U.S.A.). Rabbit polyclonal antibodies directed against aggrecan and its precursors, and the S103L monoclonal antibody have been described previously (Vertel and Dorfman, 1979; Vertel et al., 1993b). FITC-coupled *Bandeiraea simplicifolia* lectin I was obtained from Vector (Burlingame, CA, U.S.A.).

Cell culture

Chondrocytes were prepared from the sterna of 16-day-old normal White Leghorn chicken embryos and embryos carrying the genetic mutation, nanomelia, as described previously (O'Donnell et al., 1988). Embryos with the trait nanomelia were selected from crosses between chickens known to be heterogeneous for the mutation. Cell suspensions, plated at a density of 2×10^6 cells per 100-mm-diam. Petri plate in 9 ml of Ham's F-12 medium containing 10% (v/v) FCS and 1% antibiotic–antimycotic mix, were used for the isolation of RNA and biosynthetic labelling experiments. Permeabilized cells for xylosylation studies were prepared from cells cultured on gelatinized 60-mm-diam. tissue-culture dishes at a density of 2×10^6 cells per dish in 3 ml of medium. For immunofluorescence studies, cells were cultured on gelatinized, carbon-coated coverslips under the same conditions. Cultures were incubated at 37 °C in a humidified atmosphere of 95% air/5% CO_2. When indicated, BFA was used at a concentration of 5 μg/ml.

Isolation of RNA

Total RNA was prepared from day 7 cultures of normal and nanomelic chondrocytes grown in suspension. After harvesting, normal chondrocytes were digested for 12 min at 37 °C with hyaluronidase to remove extracellular aggrecan. Harvested cells were washed with PBS, quick frozen in liquid nitrogen and stored at −70 °C. RNA was extracted in acidic guanidinium isothiocyanate according to the procedure of Chomczynski and Sacchi (1987) with an initial Polytron homogenization.

Cell-free translation

Protein synthesis *in vitro* was performed with rabbit reticulocyte lysate translation kits purchased from Promega. Individual reactions contained 16 μCi of [^{35}S]methionine and 5–10 μg of total RNA per 20 μl of reaction mix, and were run under modified conditions that included incubation at 30 °C for 2 h in the presence of 0.4 mM phenylmethanesulphonyl fluoride, 1.2 mM magnesium acetate and 120 mM potassium acetate as described previously (Vertel and Hitti, 1987).

Biosynthetic labelling of intact cells, immunoprecipitation and gel electrophoresis

Biosynthetic labelling of normal and nanomelic chondrocytes from 4-day-old suspension cultures was accomplished as described previously (O'Donnell et al., 1988). Cells were pulse-labelled for 5 min at 37 °C with 100 μCi of [^{35}S]methionine and chased in 4 ml of medium containing an excess of unlabelled methionine or labelled for 2 h with 100 μCi of $Na_2{}^{35}SO_4$. Cell aliquots were removed at the indicated times, collected by centrifugation in a microfuge, washed with cold Hanks' balanced salt solution by repeated suspension and centrifugation, and treated for immunoprecipitation or for gel electrophoresis. Immunoprecipitations of cell-synthesized and cell-free translated products with S103L, and SDS/PAGE were as described previously (Vertel and Hitti, 1987; O'Donnell et al., 1988). For determinations of chondroitinase sensitivity, immunoprecipitates of radiolabelled products were resuspended in 40 μl of 0.06 M sodium acetate/0.05 M Tris, pH 7.5 containing 0.02% azide, divided into two aliquots and incubated for 3 h at 37 °C in the presence or absence of 0.25 units/ml chondroitinase. Samples were then treated for SDS/PAGE.

Xylosylation

Semi-intact cells were prepared from chondrocytes grown in monolayer culture using described permeabilization procedures and conditions for labelling (Beckers et al., 1987; Kearns et al., 1993; Vertel et al., 1993a). Briefly, cells were swollen in hypotonic buffer (15 mM KCl, 50 mM Hepes, pH 7.2) for 10 min on ice and permeabilized by scraping from the dish with a rubber policeman in 'breaking buffer' (90 mM KCl, 50 mM Hepes, pH 7.2). On the basis of the uptake of Trypan Blue, > 95% permeabilization was achieved. Semi-intact chondrocytes were labelled with 30 μCi of UDP-[^{14}C]xylose for 15 min at 37 °C.

Immunofluorescence and lectin localization

At day 5 of culture, chondrocytes in monolayer culture were fixed with 75% (v/v) ethanol and processed for immunofluorescence staining with rabbit polyclonal antibodies raised to aggrecan as previously described (Vertel and Dorfman, 1979; Vertel et al., 1993b). Immunolocalization was detected with FITC-coupled goat anti-(rabbit IgG). For lectin localization, ethanol-fixed chondrocytes were incubated with FITC-coupled *Bandeiraea simplicifolia* lectin I, which recognizes α-galactosyl residues (Hayes and Goldstein, 1974). Immuno- and lectin-labelled chondrocytes were observed and photographed under a Leitz Ortholux microscope equipped with phase and epifluorescence optics.

RESULTS

Nanomelic aggrecan core-protein-like precursor is translated directly in a truncated form

Our previous work demonstrated the synthesis of a smaller core-protein-like precursor by nanomelic chicken chondrocytes. However, those experiments did not allow us to determine whether the abnormal precursor was truly a shorter protein product or whether it resulted from an aberrant post-translational mechanism. In order to distinguish between these alternatives, we performed cell-free translation experiments with RNA prepared from normal and nanomelic chondrocytes. Northern-blot analysis confirmed the presence of low levels of normal-sized aggrecan mRNA in nanomelic chondrocytes, as reported by Stirpe et al. (1987). When RNA was used to direct protein synthesis *in vitro*, immunoprecipitable aggrecan-related precursors were detected among the products (Figure 1). The core-protein precursor, migrating as a protein of 340 kDa, was translated from RNA of normal chondrocytes (Figure 1, lanes 1 and 3). In contrast, RNA from nanomelic chondrocytes directed the synthesis of an aggrecan-related precursor that was only 280 kDa (Figure 1, lanes 4 and 6). On the basis of a comparison of electrophoretic mobilities, the nanomelic product is approx. 20% smaller than the normal aggrecan precursor. We therefore conclude that the mutant-cell-synthesized product arises directly as a truncated translation product and is not generated by post-translational events.

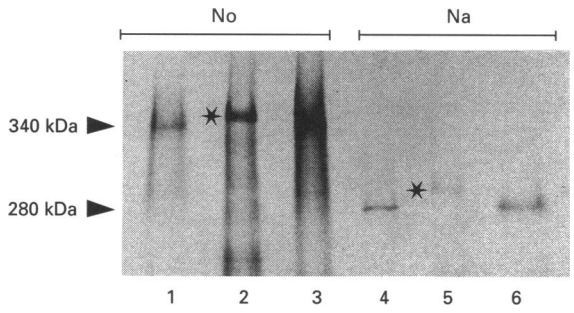

Figure 1 Both cell-synthesized and cell-free-translated aggrecan precursors are truncated in nanomelic chondrocytes

Aggrecan-related precursors were immunoprecipitated from [^{35}S]methionine-labelled products of cell-free-translation reactions directed by RNA from normal (lanes 1 and 3) and nanomelic (lanes 4 and 6) chondrocytes, and from [^{35}S]methionine pulse-labelled normal (lane 2) and nanomelic (lane 5) chondrocytes. Note that the aggrecan-related precursors of nanomelic cells (Na) (lanes 4–6) are truncated compared with the aggrecan core protein of normal chondrocytes (No) (lanes 1–3), and that the cell-synthesized precursors (lanes 2 and 5, indicated by asterisks) exhibit slightly slower electrophoretic mobilities compared with cell-free translated precursors (lanes 1, 3, 4 and 6). Products are displayed on SDS/3–5% polyacrylamide gradient gels. Positions of the cell-free-translated (arrowhead) and cell-synthesized (asterisks) aggrecan core protein precursors are indicated. Apparent molecular masses (kDa), based on the electrophoretic mobilities of protein standards, are given on the left.

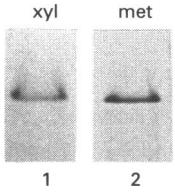

Figure 2 The truncated aggrecan precursor of nanomelic chondrocytes is xylosylated

Nanomelic chondrocytes were permeabilized, labelled with UDP-[^{14}C]xylose, immunoprecipitated with S103L and electrophoresed on SDS/3–5% polyacrylamide gradient gels as described in the Materials and methods section (lane 1). Truncated aggrecan precursor immunoprecipitated from intact cells labelled with [^{35}S]methionine is shown in lane 2 for comparison.

Truncated precursor undergoes partial processing in the nanomelic chondrocyte

Both the normal and nanomelic cell-synthesized precursors (Figure 1, lanes 2 and 5 respectively) exhibit slightly slower electrophoretic mobilities than their corresponding cell-free-translated equivalents (Figure 1, lanes 1 and 3 and lanes 4 and 6 respectively). The differences in electrophoretic mobility (approx. 20 kDa) reflect the cotranslational addition of N-linked oligosaccharides by the cells and the absence of this modification in reticulocyte lysate cell-free translation reactions. In this regard, previous studies demonstrated the endoglycosidase H sensitivity of both the cell-synthesized truncated nanomelic precursor and the normal aggrecan core-protein precursor (Vertel and Hitti, 1987; O'Donnell et al., 1988).

Recent methods developed to investigate xylose addition (Kearns et al., 1993; Vertel et al., 1993a) were applied in the study of nanomelic chondrocytes to determine whether the truncated core-protein precursor undergoes the enzymic reaction that initiates the synthesis of CS chains. Nanomelic chondrocytes were made semi-intact in order to bypass the permeability barrier normally imposed by the plasma membrane (Beckers et al., 1987; Balch, 1989), thereby permitting direct access of nucleotide sugars to intracellular organelles. When semi-intact nanomelic chondrocytes were used in radiolabelling studies, UDP-[^{14}C]xylose was incorporated into the truncated aggrecan precursor (Figure 2, lane 1). The equivalent product immunoprecipitated from intact nanomelic chondrocytes labelled with [^{35}S]methionine is shown for comparison (Figure 2, lane 2). Thus, the aberrant core-protein precursor is modified by the addition of mannose-rich oligosaccharides and xylose. Our recent studies identifying the late ER as the initial site of xylosylation in chondrocytes (Vertel et al., 1993a) are consistent with this conclusion.

Truncated aggrecan core protein can function as a substrate for CS chain elongation although it does not undergo this modification in the nanomelic chondrocyte

Experiments using the potent inhibitor BFA have played a pivotal role in elucidating the characteristics of ER-to-Golgi trafficking and distinguishing ER and Golgi functions (Klausner et al., 1992). BFA interferes with forward movement through the secretory pathway without blocking retrograde movement from the Golgi back to the ER. In essence, the Golgi collapses down on the ER, and a hybrid ER–Golgi compartment capable of both ER- and Golgi-mediated function is created in cells treated with BFA. In normal chondrocytes incubated in BFA, we observed the formation of a hybrid ER–Golgi compartment and Golgi-mediated modification of the aggrecan core protein (D. K. Mills, B. L. Grier, L. M. Walters, B. M. Vertel, unpublished work). These results suggested that in the presence of BFA, the Golgi processing enzymes of nanomelic chondrocytes would be translocated to the hybrid cytoplasmic compartment that contained the truncated aggrecan precursor (e.g. the ER) and we would be able to determine whether or not this nanomelic precursor is

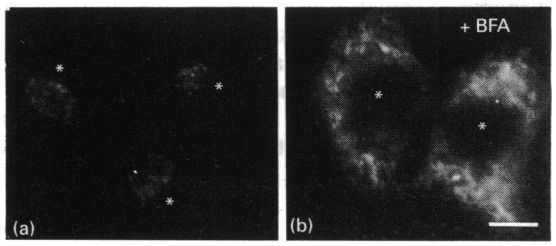

Figure 3 Treatment with BFA leads to cytoplasmic redistribution of the Golgi

Reactivity with lectin I from *Bandeiraea simplicifolia*, which is specific for α-galactosyl residues, localizes to vesicular structures in the region of the perinuclear Golgi in untreated nanomelic chondrocytes (**a**). When cells are incubated in 5 μg/ml BFA, lectin reactivity is redistributed to compartments throughout the cytoplasm and exhibits a pattern characteristic of the ER (**b**). Chondrocytes shown in this Figure were treated with BFA for 3 h before fixation. Asterisks indicate the location of nuclei. The magnification of (**a**) and (**b**) is the same; scale bar = 5 μm.

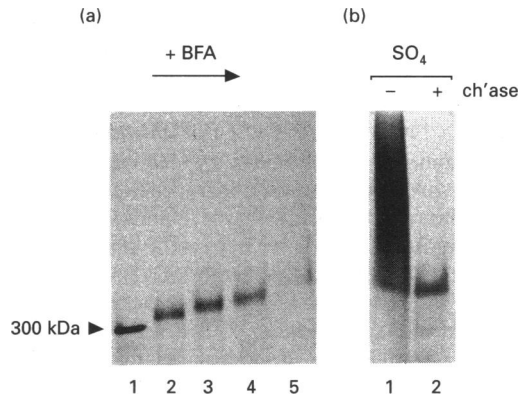

Figure 4 The truncated aggrecan precursor can be modified by Golgi-mediated processing reactions in nanomelic chondrocytes in the presence of BFA

Nanomelic chondrocytes grown in suspension culture for 4 days were preincubated for 30 min with 5 μg/ml BFA, pulse-labelled for 5 min with [^{35}S]methionine (**a**, lane 2) and chased in complete medium containing BFA for 30 min, 2.5 h and 5 h (**a**, lanes 3–5 respectively). Untreated chondrocytes pulse-labelled similarly with [^{35}S]methionine are shown for comparison (**a**, lane 1). Alternatively, BFA-treated cells were labelled with $Na_2{}^{35}SO_4$ for 2 h (**b**). At each time point, cells were collected and products immunoprecipitated with the S103L monoclonal antibody, as described in the Materials and methods section. (**b**) The immunoprecipitated products shown were incubated in the absence (lane 1) or presence (lane 2) of chondroitinase (ch'ase). All that remains of the heterogeneous, $^{35}SO_4$-labelled product (lane 1) after chondroitinase digestion is a discrete band in the region of the truncated core protein containing $^{35}SO_4$-labelled stubs of digested glycosaminoglycan chains (lane 2). Note that in chondrocytes treated with BFA, the truncated aggrecan precursor becomes larger and more heterogeneous as a function of chase time. Also the pulse sample of BFA-treated nanomelic chondrocytes (**a**, lane 2) is distinctly larger than the pulse sample of untreated cells (**a**, lane 1), suggesting that some processing steps occur relatively rapidly.

capable of functioning as a substrate for further processing. In order to verify that a reorganization of cytoplasmic organelles occurs in BFA-treated nanomelic chondrocytes, a lectin from *Bandeiraea simplicifolia*, which recognizes α-galactosyl residues, was used (Figure 3). In untreated cells, this lectin reacted with a restricted region of the perinuclear cytoplasm (Figure 3a), which has been shown in previous studies to correspond to the Golgi (Vertel et al., 1993b). After BFA treatment, lectin staining was redistributed to sites throughout the cytoplasm, in a localization

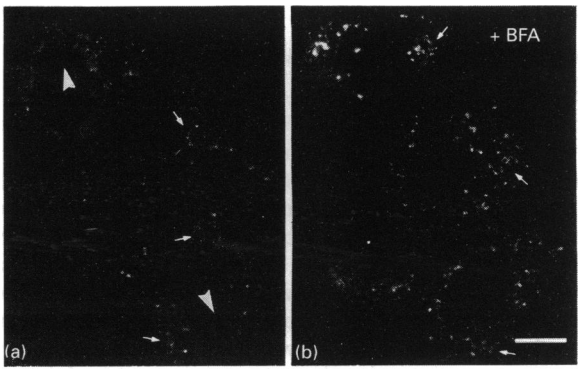

Figure 5 The subcellular distribution of mutant aggrecan precursor (e.g. the ER) remains unchanged in BFA-treated nanomelic chondrocytes

Intracellular immunoreactivity with antibodies to aggrecan and its precursors is localized in vesicles throughout the cytoplasm (small arrows) and excluded from the region of the perinuclear Golgi (arrowheads) in untreated nanomelic chondrocytes (**a**). When cells are incubated in 5 μg/ml BFA, mutant aggrecan precursors continue to be distributed in cytoplasmic vesicles, although the region of the perinuclear Golgi is no longer distinct (**b**). In this case, chondrocytes were treated with BFA for 3 h before fixation. Scale bar = 5 μm.

pattern characteristic of the ER (Figure 3b). Once the Golgi reorganization was established for nanomelic chondrocytes in BFA, biochemical experiments were initiated to evaluate the processing capabilities of the truncated precursor. We observed that in the presence of BFA, nanomelic chondrocytes convert the mutant aggrecan precursor into a product of increasing size and heterogeneity, suggestive of more extensive post-translational processing (Figure 4a). The incorporation of sulphate into the heterogeneous processed form indicates that the modification includes the addition and elongation of glycosaminoglycan chains (Figure 4b, lane 1). This conclusion is further supported by the chondroitinase sensitivity exhibited by the modified product (Figure 4b, lane 2). In Figure 5(a), immunofluorescence staining with antibodies that recognize aggrecan and its precursors demonstrates the localization of the truncated aggrecan precursor within ER subcompartments in nanomelic chondrocytes and its absence from the region of the perinuclear Golgi. In the presence of BFA, the modified product continues to reside in sites throughout the cytoplasm (Figure 5b). Thus, although the product is biochemically modified when the nanomelic chondrocyte is treated with BFA, its cytoplasmic distribution is not altered. We conclude that the nanomelic precursor is capable of being modified by Golgi-mediated processing events but, without experimental intervention, is not accessible to the required Golgi enzymes.

DISCUSSION

Experiments to date support the hypothesis that nanomelic chondrocytes produce a truncated aggrecan precursor that moves no further in the secretory pathway than the ER, and is perhaps degraded there. For example, normal chondrocytes produce a 370 kDa core-protein precursor that, with time, is converted into fully processed aggrecan, a highly modified product of $(1–5) \times 10^6$ Da. In contrast, pulse-labelling of nanomelic chondrocytes revealed a 300 kDa precursor that disappeared with time of chase. No larger processed or secreted forms were detected (O'Donnell et al., 1988). Immunolocalization studies demonstrated that the defective precursor is contained within smooth-membrane-limited subcompartments of the ER, but was never

observed in the perinuclear Golgi or in the ECM (Vertel et al., 1993b). As we show in this report, the synthesis of a truncated precursor in cell-free translation reactions is directed by RNA from nanomelic chondrocytes. This finding establishes that the defective product is specified by information encoded directly in the mRNA, and does not arise by a post-translational mechanism. The conclusion is further supported by our recent studies that define the molecular basis of nanomelia as a G to T transversion leading to a premature stop (Li et al., 1993).

Since the truncated aggrecan precursor is arrested in the ER, the nanomelic chondrocyte offers a unique model for the systematic study of compartment-specific modifications that occur during the early stages of aggrecan biosynthesis and processing. The differences in electrophoretic mobilities between the cell synthesized and cell-free translated precursors, combined with the previously reported endoglycosidase H sensitivity of the cell-synthesized products (O'Donnell et al., 1988), verify the presence of mannose-rich oligosaccharides which are known to be added co-translationally (Kornfield and Kornfield, 1985). Experiments using semi-permeabilized nanomelic chondrocytes show that the truncated precursor is xylosylated, indicating that it acts as a competent substrate for the xylosylation machinery in a manner analogous to that of normal core protein. Furthermore, as all evidence indicates that the truncated precursor is arrested in the ER, this result is consistent with the view that xylosylation occurs in the ER, and adds additional support to recent subcellular fractionation and electron microscopic autoradiography studies that show xylosylation to be a late ER-to-early Golgi-mediated process (Kearns et al., 1993; Vertel et al., 1993a).

The lack of further processing is interpreted as a failure of the truncated nanomelic precursor to be translocated to the Golgi where, presumably, processing would normally continue. Since the defective precursor never arrives in the Golgi, it is difficult to determine whether or not it is capable of being further processed. To circumvent this problem, BFA was employed. Through a mechanism that apparently interferes with vesicle coating, this inhibitor blocks forward movement from the ER to the Golgi, causes most of the Golgi to fuse with the ER, and leads to the formation of a hybrid ER–Golgi capable of many activities normally associated with Golgi function (Klausner et al., 1992). In nanomelic chondrocytes treated with BFA, the truncated precursor became modified by the addition of sulphated, chondroitinase-sensitive glycosaminoglycan chains. Although significant biochemical processing occurred, the modified mutant precursor still remained localized within cytoplasmic subcompartments characteristic of the ER (in BFA-treated cells, these sites correspond to the hybrid ER–Golgi fusion compartment). We conclude that without experimental intervention, processing fails to continue because the Golgi enzymes that mediate these reactions do not come into contact with the mutant substrate.

The comparison of size on the basis of the relative electrophoretic mobilities of the cell-synthesized and cell-free translated aggrecan precursors suggests that the truncated precursor is approx. 20% smaller than the normal aggrecan core protein, while direct comparison of coding sequences indicates that the mutant precursor is 30% smaller. This agreement is reasonable in light of the differences in experimental methods used to derive these percentages. Although it is useful to make comparisons of aggrecan core proteins (and the truncated precursor of nanomelic chondrocytes) based on masses determined from the electrophoretic mobilities of protein standards, the core-protein molecular mass of approx. 224 kDa calculated from the complete coding sequence (Li et al., 1993) indicates that these values are overestimates. Nonetheless, comparisons of either the coding sequences or the protein precursors predict that the defect is

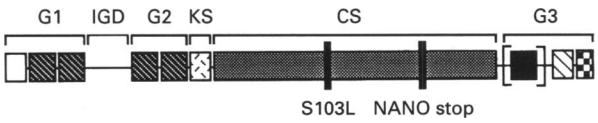

Figure 6 The domain structure of aggrecan

The domain diagram of aggrecan from Hardingham and Fosang (1992) was adapted to show the G1, G2 and G3 globular domains, and the regions of KS and CS attachment. Positions of the S103L epitope and the nanomelia translation premature stop signal (NANO stop) are indicated. The immunoglobulin fold (□) and proteoglycan tandem repeats (■) are shown within the G1 and G2 domains, as is the interglobular domain between G1 and G2 (IGD). For the G3 domain, the lectin-like domain (▨), complement regulatory protein-like domain (▩), and alternatively spliced epidermal growth factor-like domain (■, in brackets) are represented.

located in the recently reported 20-amino-acid repeat region of the aggrecan CS2 domain (see Figure 5 from Li et al., 1993).

As a consequence of the premature stop codon that we recently identified at amino acid 1513 (Li et al., 1993), the truncated precursor synthesized by the nanomelic chondrocyte is missing the entire G3 domain and the C-terminal part of the CS domain while retaining the G1, G2 and KS domains (Figure 6). The inclusion of these latter domains in the mutant precursor would suggest that the molecule maintains the following functional properties: (1) the capability of interaction with hyaluronic acid and link protein mediated by the G1 domain; (2) the capacity for N-glycosylation (most sites are located in the N-terminal domains); and (3) the potential for both CS- and KS-chain elongation. The demonstration that the molecule is modified by N-glycosylation and serves as a substrate for xylose addition indicates that the truncated precursor behaves as predicted. Moreover, the BFA experiments allow us to conclude that if the precursor were accessible to Golgi enzymes, it would also function as a substrate for glycosaminoglycan chain elongation. These results further suggest that the missing domains are not required for any of these post-translational modifications, even in an indirect fashion.

However, the fact that the truncated precursor remains stuck in the ER and is not further processed would suggest that all or part of the missing domains are in some way required for translocation and progress through the secretory pathway. In this regard, the G3 domain is of greatest interest. The function of this domain is presently unknown, but several of its properties have been investigated. For example, rotary-shadowing studies have demonstrated the globular nature of the domain (Wiedemann et al., 1984; Paulsson et al., 1987), and molecular analyses have established shared sequence identities for the N-terminal portion of the G3 domain with the hepatic lectin, and the C-terminal portion with complement regulatory protein (Sai et al., 1986; Doege et al., 1987). Specific, low-affinity interactions with several sugar ligands have been reported for the expressed domain (Halberg et al., 1988). The interpretation of these characteristics in the framework of extracellular function has led to the proposal that the domain may function in ECM assembly through interactions with carbohydrate moieties of other ECM molecules.

Although extracellular functions of the G3 domain have been emphasized in previous work, our studies suggest that intracellular functions of the missing domains need to be considered seriously. Many proteins require specific conformational changes in order to exit from the ER (Rose and Doms, 1988; Hurtley and Helenius, 1989; Pelham, 1989). The globular nature of the G3 domain indicates that it is a highly folded region of the core protein and perhaps the achievement of its native conformation

is required for movement out of the ER. Alternatively, the missing region may be involved in interactions (either with other domains or with different molecules) that must be completed before the precursor can leave the ER. As another possibility, some aspect of the missing region may function as a signal for translocation. We previously suggested that the premature stop codon might result in the exposure of an ER retention signal, but an examination of the newly created C-terminal sequence on the truncated precursor, VHETSG, shows that it bears no resemblance to the standard KDEL ER retention signal (Pelham, 1989). The possibility of the existence of other, as yet unidentified, retention or recognition signals, however, is not ruled out. It is unlikely that the epidermal growth factor-like domain is required, as this domain exists as an alternatively spliced form in the chick (Li et al., 1993) and other aggrecan mRNAs (Fülöp et al., 1993).

It has been established that retention in the ER and targeting for degradation can be the consequence of a general cellular mechanism for quality control that operates if specific conformational and assembly events fail to occur (Rose and Doms, 1988; Hurtley and Helenius, 1989; Bonifacino and Lippincott-Schwartz, 1991). Studies of viral glycoproteins and membrane receptor complexes have been particularly informative in advancing our understanding of this process. We can regard errors in the trafficking of defective cell products, such as those described for the mutant nanomelic precursor, in this context. Related mechanisms have been implicated for several other genetic diseases, including familial hypercholesterolaemia, some forms of Tay-Sachs disease, and cystic fibrosis (Amara et al., 1992). For example, one patient with Tay-Sachs disease accumulated gangliosides in lysosomes as a result of the production of a truncated precursor of the α-subunit of lysosomal β-hexosaminidase that was retained and degraded in an early biosynthetic compartment (presumably the ER) rather than being delivered to the lysosome (Lau and Neufeld, 1989).

Connective-tissue disorders may be considered similarly, as ECMs are composed of large, highly modified, strongly interactive macromolecules organized into characteristic assemblies. Many collagen defects have been associated with osteogenesis imperfecta, a number of chondrodystrophies and other diseases, and some proteoglycan defects have been suggested as well (Goetinck, 1983; Stanescu et al., 1984; Johnson, 1986; Prockop, 1990; Spranger and Martoux, 1990; Byers et al., 1991). In many cases, the swollen appearance of the ER and biochemical evidence of delayed secretion and impaired ECM deposition reflect errors in synthesis, processing and assembly. A detailed analysis of the intracellular mechanisms involved in the ER retention of abnormal type-I procollagen was reported in a recent study of fibroblasts from a patient with osteogenesis imperfecta (Chessler and Byers, 1992). Our studies demonstrate the first mutation of an aggrecan gene that exhibits similar characteristics. It is likely that further investigations of this nature will reveal more about the underlying defects and consequences of genetic diseases, and about basic biological processes.

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