Role of the C-terminal G3 Domain in Sorting and Secretion of Aggrecan Core Protein and Ubiquitin-mediated Degradation of Accumulated Mutant Precursors*

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Aggrecan is a complex multidomain macromolecule that undergoes extensive processing and post-translational modification. A thorough understanding of the events and signals that promote translocation of aggrecan through the secretory pathway is lacking. To investigate which features of the C-terminal G3 region are necessary for successful translocation of the core protein, a number of deletion constructs based on the chick aggrecan cDNA sequence were prepared and transiently expressed in COS-1 cells and the natural host, embryonic chick chondrocytes; stable cell lines were established as well. The present results clearly establish a precise requirement for that portion of the G3 C-lectin domain encoded by exon 15 for: (i) translocation from the endoplasmic reticulum (ER) to the Golgi, (ii) secretion from the cell, (iii) galactosylation of chondroitin sulfate (CS) chains, (iv) generation of Ca\(^{2+}\)-dependent galactose binding ability. Furthermore, in the absence of this subdomain there is excess accumulation in the ER of expression products leading to a stress-related response involving the chaperones Grp78 and protein disulfide isomerase, followed by degradation via a ubiquitin-proteosome pathway. All of these events in the model system faithfully mimic the naturally occurring embryonic chick chondrocytes, which also elicits a ubiquitin-mediated degradation response due to the accumulation of the truncated core protein precursor. This study represents the first report of the mode of degradation of overexpressed or misfolded proteoglycans and suggests that, although proteoglycans follow different glycosylation pathways from other glycoproteins, they are monitored by an ER surveillance system similar to that which detects other misfolded proteins.

Aggrecan, the major proteoglycan of the cartilage extracellular matrix, contains two globular domains in the N-terminal portion, G1 and G2, and one C-terminal globular domain, G3. Between the G2 and G3 globular domains, glycosaminoglycan chains (GAGs)\(^1\) are covalently attached to an extended core protein. Functionally, it is well established that the G1 domain is responsible for interaction with hyaluronan in the extracellular matrix. The C-terminal G3 domain has been implicated in synthesis and maturation of aggrecan, because its absence is associated with the avian chondrodystrophy, nanomelia. The nanomelic chick bears a mutation in the aggrecan gene that introduces an early stop codon into the translated sequence, resulting in synthesis of a truncated core protein, which is neither glycosylated nor secreted by chondrocytes (1). The mutant precursor is modified by addition of N-linked oligosaccharides and the chondroitin sulfate chain initiating xylose, but does not acquire mature CS chains, consistent with the conclusion that it progresses no further than the endoplasmic reticulum (ER) in the secretory pathway (2). These studies suggested a previously unrecognized role for the C-terminal globular domain: that it might contain recognition or retention signals or that proper folding of this specific domain, which may involve interactions with molecular chaperones, is necessary to effect exit of the entire core protein precursor from the ER. The latter mechanism would place the nanomelic mutant into the important category of folding abnormalities, which are increasingly being found to be responsible for genetic diseases, such as cystic fibrosis (3).

In attempting to determine how the accumulation of aggrecan in the ER is related to the role of the G3 domain in intracellular trafficking of proteoglycans, a few studies have expressed G3 constructs in host cells and assessed their behavior. Luo et al. (4) showed that the G3 domain is more efficient in facilitating the secretion of a CS sequence than is the N-terminal G1 domain. When exon deletion constructs were used to determine which domains were responsible for the secretion of the G3 domain, only proteins lacking the C-lectin subdomain were not secreted, whereas those lacking the EGF- and CRP-like subdomains were normally passaged; oddly, one construct (GAG5) lacking EGF, lectin-like, and CRP was also secreted (5). More recently, each lone protein module, lectin-, CRP-, or EGF-like, was shown to facilitate the translocation and secretion of a CS sequence (6). The discrepancies between these independent studies are not readily explained and none relate directly to the naturally occurring mutation in the nanomelic chick, which prevents translocation and processing of the aggrecan core protein. Therefore, we have initiated studies to better define the intracellular processing of aggrecan, focusing on the G3.

In eucaryotic cells the ER has the inherent function of endogrowth factor; CRP, complement regulatory protein; DTSSP, 3,3-dithiobis(sulfosuccinimidylpropionate); PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PDI, protein disulfide isomerase; CRD, C-type carbohydrate recognition domain.

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‡ The abbreviations used are: GAG, glycosaminoglycan chain; CS chain, chondroitin sulfate; ER, endoplasmic reticulum; EGF, epidermal growth factor; CRP, complement regulatory protein; DTSSP, 3,3-dithiobis(sulfosuccinimidylpropionate); PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PDI, protein disulfide isomerase; CRD, C-type carbohydrate recognition domain.

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suring that only correctly folded and assembled proteins are forwarded to their destinations. The term "quality control" has been adopted to describe the process of conformation-dependent molecular sorting of newly synthesized proteins in the ER (7). Most commonly, misfolded or incompletely assembled proteins are retained in the ER and eventually degraded. A number of mechanisms detailing how this is accomplished have been elucidated over the past decade, and most involve ER chaperones or folding enzymes (7).

Furthermore, under conditions that overwhelm the ER surveillance system and promote intracellular association of substrate proteins with ER chaperones like calnexin and BiP, accumulation of misfolded or aberrant proteins leads to targeting of those proteins for subsequent degradation in the cytosol, mediated by the ubiquitin-proteosome pathway (8). At present little is known about normal intracellular degradation of proteoglycan precursors or about the surveillance system that recognizes and deals with defective products. Thus, studies have also been initiated to characterize the degradative pathways and determine whether proteosomes are involved in aggrecan turnover.

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotides were made with an Applied Biosystems 3808 DNA synthesizer. The following reagents for biochemical and molecular cloning experiments were of the highest quality available and purchased from commercial vendors: restriction endonucleases from New England BioLabs, Taq polymerase from PerkinElmer Life Sciences, pTargetT mammalian expression vector and T4 DNA ligase from Promega, DTSSP (3,3-dithiobis(sulfosuccinimidyl propionate)) and β-galactose affinity resin from Pierce, anti-Grp78 and anti-ubiquitin antibodies from Stress Gen Biotechnologies, anti-PDI antibody from Affinity Bioreagents, anti-Golgi 58,000-dalton protein from Sigma.

Synthesis of Deletion Constructs—The inserts for plasmid constructs N1, N2, N3, N4, N5, and N6 (Fig. 1A) were made by PCR using the full-length NC construct as a template, each using the 5'-primer, NAP 1, TTCGTGCTCTAAACCGGATCAGTCCCTCCGAG. The NC construct contains the chick aggrecan signal peptide exon, a portion of the CS exon beginning at nucleotide 3857 which encompasses the S103L epitope, all of the G3 domain exons, and a portion (78 base pairs) of the 5'-end, until the G3 domain. Incorporation of the naturally occurring S103L mutation into the NC construct yielded six truncated species of CS as evident from the EcoRI digestions (Fig. 1B). The inserts were subcloned into the pTargetT expression vector. Clones were screened for the correct inserts by plasmid DNA isolation and subsequent Xhol/XbaI digestion for 2 h. Automated sequencing of the various constructs was done to confirm the appropriate orientation of the inserts and exclude PCR artifacts. DNA was purified using the Qiagen Plasmid Maxiprep kit.

Cell Culture and Transfection—Chondrocytes were prepared from sterna of 15-day-old chick embryos and grown in F12 media with 10% fetal calf serum supplemented with gentamicin at a density of 5 × 10⁶ cells per dish as described previously (10). COS-1 cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum at 37 °C and 5% CO₂. All transfections were performed using the calcium phosphate method (11, 12). Chondrocytes were trypsinized for 5 h before transfection and replated. COS-1 cells and chondrocytes were transfected using 10 μg of plasmid DNA. Stably transfected COS-1 cells were selected using 500 μg/ml G418. Cell lines were then maintained in media containing 250 μg/ml G418.

Immunoprecipitation and Western Blot Analysis—Media and cells were harvested and lysed in lysis buffer using a Protomixer, and proteins were separated by SDS-PAGE and transferred to nitrocellulose. The blot was blocked in 3% BSA in TBS, incubated with anti-Golgi 58,000-dalton antibody, and developed with the ECL system (Amersham Pharmacia Biotech).

Sugar Nucleotide Radiolabeling of Semi-permeable Cells—Stably transfected cell lines were made semipermeable using the permeabilization procedure of Beckers et al. (14) as modified by Kearns et al. (15). Briefly, cells were washed in hypotonic buffer (10 mM HEPES, 15 mM KCl, pH 7.2) for 10 min at 4 °C. The buffer was replaced with breaking buffer (50 mM HEPES, 90 mM KCl, pH 7.2), and cells were sheared from the dishes using a rubber policeman. Cells were pelleted at 1000 × g and resuspended in breaking buffer and incubated with 4 μCi/ml UDP-[¹⁴C]Galactose, or 250 μCi/ml [¹⁴C]UDP-galactose for 4 h at 37 °C. Labeled core proteins were immunoprecipitated and deglycosylated as described above. For autoradiography, blots or gels were incubated in amplifying solution (Amersham Pharmacia Biotech) for 20 min, dried in a gel dryer, and then processed for autoradiography (30 days, -70 °C).

Galactose Affinity Chromatography—Immunolabeled D-galactose affinity chromatography was performed as described previously (16). Cells were sheared from the dishes using a rubber policeman in hypotonic buffer and sonicated. Media and cell extracts were dialyzed against loading buffer consisting of 125 mM NaCl, 20 mM imidazole-HCl, pH 7.8, and 25 mM CaCl₂, then passed over a column packed with 2 ml of D-galactose resin (Pierce), equilibrated in the same buffer. Elution was completed in eight fractions using 64 ml of loading buffer (0.5 ml/min.). The column was rinsed between trials with 10 ml of buffer containing 2 mM EDTA substituted for CaCl₂. Fractions were dialyzed against 5 mM Tris, pH 7.4, lyopholized, and resuspended in 50 μl of chick chondroitinate digestion buffer. All fractions were treated with 0.5 unit/ml chondroitinase ABC and 0.25 unit/ml keratanase at 37 °C for 4 h. Fractions were subsequently separated on 5% SDS-polyacrylamide gels and Western blotted using S103L as the primary antibody as described above. Immunocytochemistry—Permanently transfected COS-1 cells were fixed with 4% paraformaldehyde and 0.02% glutaraldehyde and processed for indirect double immunofluorescence. The rat monoclonal antibody S103L was used to detect the expressed truncated protein products, and anti-Grp78 and anti-Golgi 58,000-dalton protein served as markers of endoplasmic reticulum and Golgi, respectively.

RESULTS

A series of deletion constructs based on the chick aggrecan sequence (1) were produced in the pTargetT expression vector (Fig. 1A). The parent sequence (NC) consisted of a portion of the CS domain, which included the S103L binding site; the EGF1 subdomain encoded by exon 13; the three subdomains of the C-lectin region, each encoded by exons 14, 15, and 16; the CRP-like domain encoded by exons 17 and 18; and a portion of the C-terminal tail (9). Each of these domains starting with the C-terminal tail was consecutively deleted from the 3'-end, until only the CS region remained, yielding six truncated species of the G3 domain. Incorporation of the naturally occurring S103L recognition site as a tool to follow the expressed proteins obviated the necessity of an artificial marker (e.g., His-tag), which might cause anomalous results (17, 18). Each construct was individually transfected into COS-1 cells as well as embryonic chick chondrocytes. Transiently transfected cultures of COS-1 cells produced fully glycosylated proteoglycans in the culture media from constructs NC, N6, N5, and N4 as evident from the molecular weight shift observed after deglycosylation with chondroitinase (Fig. 1B). In contrast, the N3, N2, and N1 (not
shown) constructs yielded almost no processed product in the media, although transfection was successful, as demonstrated by the expression of the intracellular core protein products recovered from the cell extracts. Note that N4 shows an intermediate phenotype, because, although it expresses a significant amount of intracellular precursor, the amount of product found in the media is reduced compared with that for NC-transfected cells. Also note that our results contrast with previous observations that any globular domain in the C terminus will promote translocation (6), because our N2 construct, which contains a full FGF domain, is not secreted. These results were then confirmed and extended to determine the minimal requirements for aggrecan processing and secretion.

To ensure that a similar situation pertains to the cells that produce major amount of aggrecan core protein in vivo, i.e. chondrocytes, transient transfections were performed in chick sternal chondrocyte cultures (Fig. 2). Chondrocytes transfected with empty plasmid secreted normal mature aggrecan into the culture medium, as evidenced by a >400-kDa S103L-reactive band (arrow), which appeared only after deglycosylation with chondroitinase ABC (Fig. 2, EV). This band is observed in all transfected chondrocyte lines analyzed, indicating that normal aggrecan processing and secretion occurs in the presence of the expression vector and derived constructs. The NC and N4 lane -/+ pairs (Fig. 2, Media) show that these constructs (asterisk) are expressed and secreted, mostly in a glycosylated state. In contrast, no construct-product bands are present in the Fig. 2 N3 -/+ lanes. Extracts of transfected chondrocytes (Fig. 2, Cells) contained unprocessed, native aggrecan as shown by the >400-kDa bands in the untreated lanes (arrowhead) as well as greater amounts of mature, glycosylated aggrecan in the treated lanes (arrow). The increase in the amount of normal aggrecan seen after deglycosylation suggests that three populations are present in the extracts: unprocessed, processed but not yet secreted, and secreted but attached to the cell surface. The latter fraction may be bound to cell-associated HA via the
the S103L antibody and not treated (−) or treated (+) with chondroitinase ABC. The upper panel shows immunoprecipitations from media and the lower panel those from cell extracts after SDS-PAGE and Western blot analysis with S103L antibody. The arrow indicates endogenous aggrecan. The arrowhead indicates the intracellular endogenous aggrecan precursor associated with the cell. The asterisks indicate S103L-positive expressed core proteins.

G1 domain; all of the constructs lack G1 domains and could not so attach. In contrast to the normal protein, the cell extract plasmid-expression products (Fig. 2, Cells, asterisk) show little evidence of processing. A small amount of slightly larger product may appear post-deglycosylation for constructs NC and N4, whereas no change is evident for construct N3. The major outcome of these experiments is that, although the N3 construct product clearly is synthesized in transfected chondrocytes, there is little or no secretion of the truncated N3 protein and no indication that it is glycosylated.

To ascertain where each precursor protein arrests in the processing pathway, stable cell lines prepared for N4 and N3 constructs were permeabilized, allowing labeled precursors of proteoglycan synthesis, e.g. UDP-xylene and UDP-galactose, to be incorporated into nascent core protein. Comparing the precursor-specific levels of [35S]methionine labeling in each of the expressed products, it is seen that the accumulated N3 core protein incorporates xylose but not galactose (Fig. 3). In contrast, the N4 core protein incorporates both xylose and galactose, indicating that the N4/N3 truncation interval is crucial for galactosylation. Previous studies have shown that these two glycosyltransferase activities are localized in different compartments (15, 19). Note that galactosylation in N4-expressed protein is evident after chondroitinase treatment, in agreement with the observations that xylosylation is a rate-limiting step in GAG biosynthesis and that, after translocation of the core protein to the Golgi, successive reactions proceed quickly without allowing observation of partial intermediate forms (10).

The present results imply that, until the precursor is translocated from the xylosylation-competent compartment to the next one, the core protein cannot be galactosylated, and that translocation requires information inherent in the second lectin exon. The biochemical localization of the various truncated species was confirmed by immunocytochemistry using the S103L antibody to stain stably transfected COS-1 cells. In NC- and N4-expressing cells, very little intracellular-expressed product was detected, and that which was present appeared more Golgi-localized as confirmed by double staining with the anti-Golgi 58k protein (Fig. 4, NC and N4). The truncated N3 product localized to the ER and ER-Golgi intermediate compartment as indicated by its similar distribution with the ER marker, Grp78 (Fig. 4, N3), thus confirming the biochemical evidence that deletion of the second lectin domain leads to disruption of the CS biosynthetic pathway.

To determine whether the C-lectin subdomain has functional properties that contribute to the G3 routing specificity, we examined the lectin-binding ability of the various constructs on galactose affinity columns. Culture media and cell extracts from NC, N4, and N3 stable cell lines were loaded onto a galactose affinity column (Fig. 5), as described previously (16). The Ca2+-dependent affinity for galactose was determined by the retention of the protein constructs on the column. No binding to the column was observed with any constructs in the absence of Ca2+ (data not shown). As expected, the NC core protein released into the culture medium as well as that associated with the cells, binds efficiently to the galactose column. N4 core protein released to the medium or from cell extracts also binds to the column, although not as efficiently as NC. In contrast, the N3 core protein, which lacks two thirds of the lectin-like domain and remains intracellular (see Fig. 5, Cell extract), does not bind to the galactose column. These results directly correlate the acquisition of Ca2+-dependent galactose-binding ability, which presumably reflects the presence of a specific structural motif, with intracellular G3 routing. It should be noted that the lectin-like domain contains 6 cysteine residues, 3 in exon 14, none in exon 15 and 3 in exon 16. Thus unpaired cysteines are not responsible for the misfolded lectin-like domain, because both the N3 and N4 constructs contain the same number of cysteines.

Using the stably transfected cell lines for NC, N4, and N3, we established that a cellular response to protein misfolding in the ER is triggered in the cells expressing the N3 construct. Fig. 6 shows that the ER resident proteins Grp78 (BiP) and PDI (protein disulfide isomerase) coprecipitate with S103L-reactive intracellular truncated N3 core protein, but not with S103L-positive NC or N4 core protein. We were also able to coprecipitate PDI with the NC core protein if the cells were permeabilized and incubated with DTSSP prior to immunoprecipitation (data not shown), indicating that the PDI chaperone may participate in the normal folding of the G3 domain as well. The multiple species observed in the overloaded gels of the S103L

**FIG. 2.** Expression and secretion of G3 deletion constructs expressed in primary chondrocyte cultures. E14 sternal chondrocyte cultures were transiently transfected with NC, N4, and N3 constructs and empty vector (EV) using the calcium phosphate method. After 24 h in culture the medium and cell extracts were immunoprecipitated using the S103L antibody and not treated (−) or treated (+) with chondroitinase ABC. The upper panel shows immunoprecipitations from media and the lower panel those from cell extracts after SDS-PAGE and Western blot analysis with S103L antibody. The arrow indicates endogenous aggrecan. The arrowhead indicates the intracellular endogenous aggrecan precursor associated with the cell. The asterisks indicate S103L-positive expressed core proteins.
immunoprecipitates from the transfected cell lines (Fig. 6, S103L) led us to ascertain whether the misfolded N3 core protein is degraded and being targeted for removal via the ubiquitin-proteosome pathway. To this end, we first determined whether the core protein is a substrate for ubiquitination by immunoprecipitating N3, N4, and NC core proteins from cell extracts and preparing Western blots probed with anti-ubiquitin as primary antibody. Fig. 7A shows that N3 core protein is ubiquitinated (arrowhead), suggesting that the ubiquitin-proteosome pathway is involved in the degradation of this ER-accumulated, truncated proteoglycan core protein. When larger amounts of cell extracts were immunoprecipitated (Fig. 7B) some ubiquitination can also be detected associated with the NC and N4 core proteins, which may be the consequence of overwhelming the ER surveillance system in the overexpressing cell lines. Furthermore, expression of poly-ubiquitinated N3 core protein occurs, as indicated by the presence of higher molecular weight species of S103L-positive N3 core protein (Fig. 7B, arrowhead). To determine whether the truncated aggrecan core protein expressed in the nanomelic chick undergoes a similar fate, an experiment using cartilage protein from wild type and nanomelic chicks was performed. In the nanomelic chick, the accumulated intracellular core protein is ubiquitinated (Fig. 7C), whereas the aggrecan core protein from normal cartilage also shows some level of ubiquitination, suggesting that the ubiquitin-proteosome system is involved both in removal of the ER-accumulated, nanomelic precursor, as well as in the normal processing of misfolded aggrecan core protein.

**DISCUSSION**

From our studies, which elucidated the mutation in the nanomelic chick, it was learned that not only was there a complete lack of aggrecan in the mutant extracellular matrix, there was also an accumulation of a truncated, partially processed precursor in the chondrocyte ER, leading to the hypoth-
thesis that the deleted G3 domain was involved in the intracellular maturation and secretion process of this proteoglycan (1, 2). In the present study, we focused mainly on: (i) identifying the minimal alteration from the native structure tolerated before aggrecan processing and secretion are affected, (ii) elucidating the pathway and players involved in the early steps of processing, (iii) investigating whether the unnatural accumulation of unprocessed core protein induces a stress-related response, and (iv) determining the mode of degradation of the ER-accumulated product.

To examine the importance of the G3 domain for successful translocation from the ER to the ensuing compartments of the secretory pathway, a series of deletion constructs based on the chick sequence were prepared and tested on model cell lines (COS-1 cells) as well as on cell types that predominantly manufacture aggrecan (primary chick chondrocytes). The latter is important, because it is known that the secretory pathway is differentiated in specific cell types and it is therefore not assured that a protein will fold properly in all cell types (7). Perhaps this explains some of the unusual findings of the two previous studies, both of which were done in non-chondrocytic cell lines (5, 6). Although not differing qualitatively, our studies do indicate that chondrocytes are much more efficient in processing the constructs than COS-1 cells, highlighting the presence of a more highly developed or specific system for processing this complex product in chondrocytes.

The translocation-competence properties of the G3 subdomains were then assessed by an exon-by-exon dissection of the G3 domain followed by assays for compartmental transfer, functionality, and modes of degradation. Experiments designed to determine what is recognized in the G3 domain that triggers forward transport indicate that peptide sequence N-terminal to and including the second C-lectin subdomain is required for intracellular translocation of the chick aggrecan constructs (Fig. 2), because there was abundant product secreted into the media for the N4 construct (which is missing the last exon of the lectin domain) and no secretion for the N3 construct (missing the last two lectin exons). That there is a distinct recogni-
tion element encoded by the middle lectin-like exon required for movement out of the ER and further steps of glycosylation was determined by defining the ability of the N3 and N4 constructs to be xylosylated and galactosylated. We previously developed this methodology using semipermeable cells and direct labeling with the immediate nucleotide precursors to define the topography (location and organization) of the numerous glycosyltransferase reactions required for synthesis of CS chains on proteoglycans (15). The present results indicate that the N3 construct indeed remains localized to the ER compartment, because only xylose was incorporated into it, whereas the N4 construct presumably was competent to proceed to the next series of glycosyltransferase reactions exemplified by galactose addition in the Golgi compartment. That the presence of a structural motif so clearly demarcates the translocation from late ER to cis-Golgi, as illustrated by lack of galactosylation, suggests a requirement for this motif in the folding process of the core protein into a conformation that is allowed to exit the ER surveillance apparatus (7, 20). In fact, part of this surveillance system may be the CS-chain-initiating xylosyltransferase, which has been hypothesized to play a role in processing and translocation from the ER of glycosylated proteoglycans such as aggrecan (21).

Also suggestive of a functional conformation imposed on the aggrecan core protein by the second lectin domain is the observed ability to generate a galactose-binding motif. To determine whether the C-lectin subdomain has functional properties that contribute to the G3 routing specificity, we examined the lectin-binding ability of the various expressed proteins on galactose affinity columns. The expressed and secreted N4 product was fully capable of calcium-dependent binding to a galactose column, whereas N3 was not, indicating that the second but not the third C-lectin domain exon is required to generate a galactose binding motif. These results are surprising, because the structural basis for galactose recognition by C-type animal lectins described in the literature is mainly localized in the third exon of this domain (22–24); thus in the absence of the third exon, an alternative galactose-binding site may be generated that allows some calcium-dependent binding ability to be maintained. These C-type carbohydrate recognition domains (CRDs) have been extensively studied in the aggrecan core protein where Ca\(^{2+}\)-dependent sugar binding by this region has been demonstrated (16, 25). It has been speculated that this sugar binding ability is related to organization of the proteoglycan in the extracellular matrix. At the least, our results indicate that acquisition of this common structural motif is required for translocation from the ER to the Golgi and further processing of the core protein, and perhaps for later functions in the extracellular matrix as well. Although these results suggest that a folding defect occurs that does not allow further processing of the core protein when the second and third exons of N3 are absent, it is also possible that, as has been described in other instances (26, 27), the selective incorporation of the core protein into COPII vesicles, which bud off from the ER, is regulated by binding of small molecules to the CRD domain. Although speculative at present, it remains an attractive possibility in light of the fact that this set of core proteins should be directed to a subset of specific glycosyltransferases in the Golgi and that the truncated products that do move forward in the secretion pathway maintain a functional CRD domain.

It was established that the intracellular accumulation of abnormally folded or aggregated protein activates the cellular stress response, often leading to attenuation of the cell’s ability to tolerate subsequent stress and thus to premature cell death via apoptosis (28). Using stably transfected cell lines, which accumulate the modified aggrecan G3 constructs in the ER, we examined whether a stress response is mounted, as has been shown for other disease processes (7). To verify that certain chaperones or folding factors (both known and novel) participate in the quality control of aggrecan processing in the ER, co-immunoprecipitation experiments with S103L followed by Western blots with antibodies to Grp78 and PDI indicate that these two chaperones were co-immunoprecipitated with accumulated N3 precursor. These associations may result from an effort of the surveillance system to refold the misfolded N3 core protein or from a hydrophobic motif being exposed in the truncated N3 construct that promotes a prolonged interaction with these chaperones. Because PDI can also be immunoprecipitated with NC and N4 core proteins after cross-linking, it is possible that this chaperone is involved in the normal processing of this core protein as well. These results suggest that, although proteoglycan core proteins follow quite different glycosylation pathways from other glycoproteins, they are monitored by an ER surveillance system similar to that which detects other misfolded proteins.

Accumulation of misfolded or aberrant proteins can also lead to targeting of those proteins for subsequent degradation in the cytosol mediated by the ubiquitin-proteosome pathway (8, 20). Co-immunoprecipitation experiments demonstrated specific ubiquitination of both the N3 product and the truncated nanomelic aggrecan, providing initial evidence for proteosomal degradation of aberrant proteoglycan core proteins. The fact that ubiquitination is also observed with the NC and N4 core proteins, as well as with the normal aggrecan core protein, suggests that the ubiquitin-proteosome pathway is the normal processing route for overexpressed or misfolded aggrecan core protein in the ER. However, in the N3-expressing cell line ubiquitination occurred at higher levels, as indicated by the detection of a ladder of ubiquitinated N3 core proteins, even in the absence of proteosome inhibitors (8).

Lastly, the phenotypic characteristics of the N3 (and further-deleted) generated proteins are comparable to the behavior of the truncated aggrecan core protein expressed in the nanomelic mutant, at both the biochemical and trafficking levels, because it has been established that the nanomelic aggrecan core protein is halted in the ER compartment where it is xylosylated but not further processed (1, 29). In the present work we have shown that the abnormally accumulated product in the nanomelic chondrocyte is likely dealt with via the ubiquitin-proteosome pathway, as is the accumulated core protein in the N3 expressing cell lines. It also could be predicted that concomitant with degradation there is a stress response elicited in the nanomeric chondrocyte contributing to the overall phenotype of the mutant. Future studies will address this possibility.

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