Divergent Secretory Behavior of the Opposite Ends of Aggrecan*

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The proteoglycan, aggrecan, has a globular domain, G1, at the N terminus and a different globular domain, G3, at the C terminus. Aggrecan produced by mutant nanomelic chickens is truncated due to a premature stop codon and consequently lacks G3 and a minor portion of its chondroitin sulfate domain (Li, H., Schwartz, N. B., and Veltel, B. M. (1993) J. Biol. Chem. 268, 23504-23511). The mutant protein is retained in the endoplasmic reticulum and fails to enter the Golgi stacks (Vertel, B. M., Walters, L. M., Grier, B., Maine, N., and Goetinck, P. F. (1993) J. Cell Sci. 104, 939-948). The homozygous mutant is lethal because of failure of chondrogenesis and osteogenesis, while the heterozygous mutant is dwarfed. To further elucidate the pathogenetic mechanisms underlying nanomelia and to determine if G1 and G3 are themselves secreted, we expressed them in transfected host cells. Expression was performed in wild type Chinese hamster ovary (CHO) cells and in mutant CHO cells which are unable to link glycosaminoglycan (GAG) chains to core proteins. We compared: (a) secretion of expressed G1 and G3 constructs containing contiguous GAG chain consensus sites and (b) GAG chain modification of the secreted proteins. We find that: 1) G3 is 24-100 times more rapidly secreted than G1; 2) secreted G3 contains contiguous chondroitin sulfate GAG chains, while secreted G1 lacks contiguous GAG chains; 3) G3 secretion is not coupled to xylosylation of contiguous GAG chain consensus sites. These results imply that G1 and G3 intrinsically differ in passage through the cell secretory route.

The secretory pathway of a protein destined for cell export requires at least four components (1): (a) recognition elements for nascent protein insertion into the ER, (b) molecular chaperone surveillance mechanisms, (c) endogenous enzymes for post-translational modifications, and (d) routing procedures to deliver the finished protein to the cell exterior. Mutant proteins whose normal forms are ordinarily destined for cell export often fail to navigate the secretory pathway and are retained within cells (2). One proposed explanation for retention is that mutant proteins have abnormal conformations and fail to pass surveillance by molecular chaperones (3). The chaperones primarily reside in the ER lumen, where they are gatekeepers for secretory protein entry into the Golgi stacks. Such entry requires proper conformation of the globular domains of a core protein. Improperly folded proteins are either retained in the ER lumen until they become properly folded or they are degraded.

Aggrecan contains three globular domains, G1, G2, and G3, with contiguous consensus sites between G2 and G3 for attachment of keratan sulfate and chondroitin sulfate chains (4). Aggrecan is normally secreted into the extracellular matrix, where it is responsible for tissue resiliency. Its presence is vital for normal chondrogenesis and osteogenesis. G3 is absent in nanomelia due to a premature stop codon (5, 6); the truncated aggrecan is not secreted and accumulates in the ER lumen (7). The homozygous mutation is lethal, while the heterozygote survives as a dwarf animal.

Members of a proteoglycan family comprised of aggrecan, versican, neurocan, and brevican share homologous G1 and G3 domains (8). G3 has three subdomains one of which, a C-lectin, is highly conserved and occurs in a wide variety of secreted and cell surface proteins (9). G3 is often absent from secreted aggrecan (10), suggesting that G3 may be dispensable in the extracellular space. In contrast, G1 is retained in secreted aggrecan and has the important role of binding to hyaluronan, producing an extracellular polymeric network (11, 12). The present study was designed to focus on the behavior of G1 and G3 when they have been introduced into the secretory pathway of host cells, which normally produce proteoglycans. The results imply that G1 and G3 behave very differently from each other in the secretory pathway, suggesting that they may have separate roles in intracellular routing of aggrecan.

EXPERIMENTAL PROCEDURES

Construct Cloning—1) The G3 construct contains the endogenous avian aggrecan signal peptide (SP), endogenous avian glycosaminoglycan consensus sites (GAG), endogenous avian C-terminal G3 domain (G3) (13, 14) and a 6-histidine (His6) tag, abbreviated as SP-GAG-G3-His6. SP was amplified by polymerase chain reaction (PCR) using oligonucleotides 101 (5′-GACGTCGGATCCAT-3′), 16447 containing a HindIII site and 102 (5′-CTGCAGTCTAGAT-3′), containing an XbaI site. The GAG and G3 sequences were amplified by PCR using oligonucleotides 103 (5′-CTGCTGAGCCTTTCTGAAAATT-3′), containing an XbaI site and 104 (5′-GACGTCGGATCCAT-3′), containing an XhoI site, stop codon, and His6 tag. PCR products were first digested with XbaI and then ligated into HindIII and XhoI sites of pcDNA3 vector (Invitrogen). 2) The G1 construct contains SP, endogenous N-terminal G1 domain (G1), GAG, and His6 tag, abbreviated as SP-G1-GAG-His6. The SP, GAG, and His6 domains are identical to those of construct G3. SP and G1 sequences were amplified by PCR using oligonucleotides 203 (5′-GACGTCGGATCCAT-3′), containing a BamHI site and 201 (5′-GACGTCGGATCCAT-3′), containing an EcoRI site and 103 (5′-CTGCTGAGCCTTTCTGAAAATT-3′), containing an XbaI site. The GAG and G3 sequences were amplified by PCR using oligonucleotides 203 (5′-GACGTCGGATCCAT-3′), containing a BamHI site and 201 (5′-GACGTCGGATCCAT-3′), containing an EcoRI site and 104 (5′-GACGTCGGATCCAT-3′), containing an XhoI site, stop codon, and His6 tag. PCR products were first digested with XbaI and then ligated into HindIII and XhoI sites of pcDNA3 vector (Invitrogen).
Aggrekan Domain Secretion

RESULTS

This study was designed to elucidate whether globular domains G1 and G3 are themselves able to transit through the secretory pathway. They were placed in constructs that reflect their arrangement in aggrecan itself. Fig. 1 shows the linear arrangement of the components and deduced amino acid sequences of the G1 and G3 constructs. Included are the endogenous avian aggrekan signal peptide, endogenous avian GAG consensus sites, and a C-terminal 6-histidine (His6) tag. The endogenous avian G1 and G3 domains are present in the respective constructs, which are abbreviated as SP-G1-GAG-His6 and SP-GAG-G3-His6, respectively.

Fig. 1. Diagram of G1 and G3 domain organization in their respective constructs and the corresponding amino acid sequences. a, G1 construct; b, G3 construct. Both constructs contain the endogenous avian aggrekan signal peptide, endogenous avian GAG consensus sites, and a C-terminal 6-histidine (His6) tag. The endogenous avian G1 and G3 domains are present in the respective constructs, which are abbreviated as SP-G1-GAG-His6 and SP-GAG-G3-His6, respectively.

The eluates of digested proteins were electrophoresed in a 5–15% gradient SDS-PAGE gel, which was dried and exposed to x-ray film.

Chondroitinase ABC and Heparitinase I Digestion—The eluate was incubated for 2 h in the following: 1) chondroitinase ABC buffer (750 mM NaCl, 0.1% NP-40, 10 mM CH₃COONa, pH 8.0) alone; 2) chondroitinase ABC (Seikagaku) in chondroitinase buffer; 3) heparitinase buffer (100 mM CH₃COONa, 10 mM (CH₃COO)₂Ca, pH 6.2) alone; 4) heparitinase I (Seikagaku) in heparitinase buffer; 5) sequential digests done with heparitinase I in heparitinase buffer, followed by chondroitinase ABC in chondroitinase buffer. Buffer change was accomplished by using Microcon (Amicon) centrifugation.

Kinetic Experiments and Radioactivity Quantiﬁcation—Samples were collected at 4, 8, and 12 h after adding radioactive isotope. The radioactive protein bands were measured by an Instant Imager (Packard); G1 and G3 signals were corrected by subtracting background due to proteins resulting from empty vector transfection. Samples loaded onto gels were known portions of total cell lysate or medium. Total amounts of radioactive G1 and G3 in each sample were calculated by normalizing to the amounts of proteins digested with chondroitinase ABC in the harvested samples. The secretion of domains G1 and G3 is not labeled by radioactive sulfate (Fig. 2a) and its se...
host cells, which are unable to add GAG chains to core proteins, migrate near 82 kDa. Transfection of two different mutant GAGs anomalously in SDS-PAGE gels, each appearing about 2-fold larger than predicted by amino acid composition (Fig. 1). Intracellular G1 migrates near 91 kDa, while intracellular G3 migrates near 82 kDa. Transfection of two different mutant host cells, which are unable to add GAG chains to core proteins (17), confirmed the apparent sizes of G1 and G3 core proteins (Fig. 3). Core protein anomalous migration may be due to the His-termination tag, which seems to cause this behavior in other expressed proteins (18).

The pgsA and pgsB mutant cell lines are defective in xylosyl transferase and galactosyl transferase, respectively, the first two enzymes which catalyze xylose and galactose addition to core proteins. These two sugars form part of the linkage between core proteins and CS/HS chains. The G3 protein in the medium of these mutant cells lacks GAG chains and is similar in size to the smallest G3 components of the nonproteoglycan population produced by wild type cells (Figs. 2-4). Such small non-sulfated G3 neproteoglycan species, secreted from wild type host cells, may have relatively few or incomplete GAG chains, perhaps due to saturation of one or more post-translational enzymes by excess core protein. Alternatively, they may have been secreted via a "shunt" pathway, bypassing the Golgi stacks.

Secretion was assessed by measuring total radioactivity in intracellular and extracellular G1 and G3 populations. After overnight labeling of wild type and mutant cells, G3 was only detectable in the medium, whereas G1 was discernible in cell extracts and medium (Fig. 3). G3 was detectable in cells after shorter labeling periods (Fig. 4); its level in lanes 4 and 5 is readily measured above the corresponding background in lanes 1 and 2. Kinetic analysis confirmed that G3 secretion from cells is greater than G1 secretion; the difference was 24-fold at 4 h, 48-fold at 8 h, and 100-fold at 12 h. This progressive increase probably reflects accumulation of G1 inside the cells. The rate of secretion of G3 is clearly quite different from that of G1.

It is not known whether aggrecan core protein requires xylosylation for exit from the ER to the Golgi stacks (19). Our results show that a partial aggrecan, based on G3, is readily secreted in the absence of xylosylation when transfected into pgsA CHO cells (lanes 1–3), mutant pgsB CHO cells (lanes 4–6), and mutant pgsB CHO cells (lanes 7–9), respectively. Mutant pgsA cells lack xylosyl transferase and mutant pgsB cells lack galactosyl transferase I; xylosyl transferase catalyzes addition of xylose to serines in GAG chain consensus sites, while galactosyl transferase I catalyzes addition of galactose to xylosylated sites. These two carbohydrates are part of the tetrasaccharide unit, which links core proteins to CS/HS chains. The bracket indicates G3-derived neoproteoglycan in lane 2, while G3 and G1 core proteins are designated by arrows. Note that the entire G3 population in lane 2 is as abundant as G3 without xylose addition (lane 5) or G3 without galactose addition (lane 8). b, cell lysates; autoradiography of empty vector proteins, G3 and G1 proteins from wild type CHO cells (lanes 1–3), mutant pgsA CHO cells (lanes 4–6), and mutant pgsB CHO cells (lanes 7–9), respectively. G3 co-migrates with an endogenous CHO protein and is not detectable in the cells after overnight labeling (lanes 2, 5, and 8), whereas G1 is visible (lanes 3, 6, and 9). The samples in panel a were exposed to film longer than the ones in panel b.
DISCUSSION

The difference in secretory behavior between G1 and G3 is consistent with retention of truncated aggrecan core protein, which lacks G3, in nanomelic chondrocytes. The two constructs used in this study represent partial aggrecan proteins, although the tandem arrangement of included domains corresponds to those of whole aggrecan itself. Not only is G1 secreted more slowly than G3 but it fails to become decorated with GAG chains. This latter observation suggests G1 either does not transit through the same secretory pathway as G3 or its consensus sites are inaccessible for GAG chain modification. In contrast, G3 is completely secreted within 16 h after transfection and a major portion of the protein is decorated with GAG chains, appearing in SDS-PAGE gels as a typical proteoglycan. Thus, a core protein encompassing G3 plus contiguous GAG chain consensus sites is able to move through the normal secretory pathway, mimicking the translocation of whole aggrecan protein; as in aggrecan protein itself, the GAG consensus sites serve as Golgi reporter groups.

In contrast, only small amounts of a protein encompassing G1 plus the same contiguous GAG chain consensus sites, which lacked GAG chains, appear in spent media. Perhaps this protein is released from dead or dying cells and had not traversed the normal secretory pathway. Alternatively, it might have traversed a “shunt” pathway, bypassing the Golgi stacks, if such exists in normal cells. G1 and G3 domains, in both aggrecan protein and the construct proteins, have opposite orientations to the contiguous GAG sites. Possibly, the tandem location site of a globular domain as C terminus versus N terminus to the GAG consensus sites may be important for traversing the secretory pathway. We do not consider this to be a likely possibility because earlier studies have shown that similar GAG chain consensus sites, flanked at the N terminus by a signal sequence and protein A, did transit through the Golgi stacks (20, 21). Those studies also showed that a C-terminal His6 tag did not affect GAG chain attachment.

Xylosylation of GAG chain consensus sites may be important for proteoglycan secretion (19, 22). Our results show that xylosylation of contiguous GAG sites is not required for G3 protein secretion; non-xylosylated G3 protein is secreted in equal amounts to xylosylated G3 protein. This result also implies that GAG chain attachment per se is not required for core protein secretion. Furthermore, our results are consistent with the concept that ER molecular chaperones primarily recognize globular protein domains (3); non-globular domains such as GAG chain consensus sites may not be subject to chaperone surveillance.

In the case of a proteoglycan with multiple globular domains, such as members of the aggrecan family, each domain is potentially subject to chaperone surveillance. It is difficult to visualize, for an individual molecule, how such multidomain surveillance might be orchestrated and regulated. The observation that domain G3 is secreted normally, while domain G1 is not, suggests that one intramolecular domain may be preferably recognized by molecular chaperones. Thus, G3 may facilitate movement of the entire aggrecan core protein through the secretory pathway. If so, it is intriguing to speculate what molecular mechanisms may account for such behavior.

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