Aggrecan Synthesis and Secretion

A PARADIGM FOR MOLECULAR AND CELLULAR COORDINATION OF MULTIGLOBULAR PROTEIN FOLDING AND INTRACELLULAR TRAFFICKING

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Each globular domain of exported multiglobular proteins putatively undergoes chaperone surveillance in the endoplasmic reticulum lumen. It is difficult to visualize how surveillance of multiple globular domains might be orchestrated and regulated. Aggrecan core protein has been used as a prototype for this problem by examining transfection of informative constructs into Chinese hamster ovary cells. The salient results are as follows: 1) aggrecan’s N-terminal G1 domain is minimally secreted, and its flanking Golgi reporter sites are not decorated with glycosaminoglycan chains; in contrast, its C-terminal G3 domain is readily secreted with flanking GAG chains, and G3 also facilitates G1 secretion; 2) G3 but not G1 can be intracellularly cross-linked to chaperone Hsp25; 3) G3 and Hsp25 remain noncovalently bound and arc secreted together when G3 is situated N-terminal to its normal location; 4) exon 15, which encodes the center of G3’s C-lectin subdomain, is necessary and sufficient for G3 secretion. A model is proposed in which Hsp25 piggybacks onto nascent G3 in the cytosol during a translocational pause and enters the ER lumen with G3, and once G3 properly folds, Hsp25 releases G3 and recycles to the nucleus while G3 continues to the Golgi stacks, providing passage for the entire core protein.

Globular proteins that are destined for export or for transit to the cell surface putatively undergo surveillance by molecular chaperones in the lumen of the endoplasmic reticulum (1, 2). This quality control mechanism ensures that globular proteins have achieved proper conformation prior to their progression in the routing process from the endoplasmic reticulum to the Golgi stacks. In the case of proteins with two or more globular domains, each individual domain is potentially subject to chaperone surveillance. It is difficult to visualize, for an individual molecule, how surveillance of multiple globular domains might be orchestrated and regulated.

Insight into this problem of chaperone surveillance of multiglobular proteins may be achieved using aggrecan as a model system. The aggrecan family of secreted proteoglycans has various members with similar molecular features (3). The N-terminal globular G1 domains of the family members are highly conserved, are virtually identical, and are lectins that bind hyaluronic acid, producing aggregates in situ (4). The C-terminal globular G3 domains are also highly conserved, are virtually identical, and contain C-lectin domains that bind monosaccharides (5) and also bind tenascin-R by calcium-mediated, non-lectin interactions (6). Thus, the C-lectin domain has at least one site for protein recognition and binding.

The importance of the G3 domain for aggrecan secretion is exemplified by the nanomelic mutation in which mutant aggrecan core protein, lacking G3, fails to leave the cell and accumulates in the lumen of the ER (7). The mutant protein is truncated due to a premature stop codon and lacks both G3 and a portion of the adjacent chondroitin sulfate consensus region (8). Transfection with various constructs has shown that it is G3, and not G1 or the chondroitin sulfate consensus domain, that is critical for aggrecan core protein intracellular trafficking and aggrecan secretion (9). The present study was designed to further examine this role of G3 in modulating aggrecan core protein intracellular routing.

EXPERIMENTAL PROCEDURES

Construct Assembly (Figs. 1 and 2)—The G1-glycosaminoglycan (GAG)3-G3 miniaggrecan construct contains the endogenous avian aggrecan signal peptide (SP), endogenous avian N-terminal G1 domain, endogenous glycosaminoglycan consensus sites, endogenous avian C-terminal G3 domain, and a six-histidine (His6) tag and is abbreviated SP-G1-GAG-G3-His6. SP was amplified by polymerase chain reaction (PCR) using oligonucleotide 203 (Table I) plus oligonucleotide 201. The GAG-G3 sequence was amplified by PCR using oligonucleotide 202 plus oligonucleotide 204, which contains His6 and a stop codon. PCR products were reamplified by overlapping PCR using oligonucleotides 203 and 104 to obtain full-length SP-G1-GAG-G3-His6, which was digested with SrfI and ligated to predigested pCR-Script SK(+) vector (Stratagene), which was then used to transform Escherichia coli XL1-blue MRF' Kan supercompetent cells. E. coli colonies were screened, and suitable inserts were released by BamHI and XhoI and then ligated into corresponding sites of the pcDNA3 vector (Invitrogen).

A contralateral G1 construct contains SP, endogenous GAG, N-terminal G1 domain, and His6, and is abbreviated SP-GAG-G1-His6. SP-GAG was amplified by PCR using oligonucleotide 203 plus oligonucleotide 302. G1 was amplified by PCR using oligonucleotide 204 plus oligonucleotide 104, which contains His6 and a stop codon. PCR products were digested with XhoI and then ligated into pCR-Script SK(+) and redigested with BamHI and XhoI and ligated into the pcDNA3 vector.

A contralateral G3 construct contains SP, endogenous C-terminal G3 domain, GAG, and His6, and is abbreviated SP-G3-GAG-His6. SP was amplified by PCR using oligonucleotide 101 plus oligonucleotide 117. G3 was amplified by PCR using oligonucleotide 118 plus oligonucleotide 119, and SP-G3 was then reamplified by overlapping PCR using oligonucleotides 101 and 119. GAG was amplified by PCR using oligonucleotide 118 plus oligonucleotide 111, which contains His6 and a stop codon. SP-G3-GAG-

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1 The abbreviations used are: GAG, glycosaminoglycan; CHO, Chinese hamster ovary cells; ER, endoplasmic reticulum; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; SP, signal peptide; Bicine, N,N-bis(2-hydroxyethyl)glycine; DTSSP, 3,3'-dithiobis[sulfo succinimidyl] propionate).
His6 was then reamplified by overlapping PCR using oligonucleotides 101 and 111. The final PCR products were digested with HindIII and XhoI and ligated into the pCDNA3 vector.

G3 and G1 constructs were produced as described by Luo et al. (9).

Exon combination constructs were made with relevant oligonucleotides (Table II) as follows. The P1 construct was amplified by PCR with the SP-G1-GAG-G3 construct as a template using oligonucleotides 203 and 401, which contains His6 and a stop codon. The P2 construct was amplified by PCR with the SP-G1-GAG-G3 construct as a template using oligonucleotides 203 and 402, which contains His6 and a stop codon. The P3 construct was amplified by PCR with the SP-G1-GAG-G3 construct as a template using oligonucleotides 203 and 403, which contains His6 and a stop codon. The P1, P2, and P3 constructs were individually digested with BamHI and XhoI and then ligated into pcDNA3 vector.

For the P4 construct, SP-G1 sequence was amplified by PCR using oligonucleotides 203 and 409. These PCR products were digested with XhoI and then ligated into the pcDNA3 vector. The GAG consensus sequence was amplified by PCR using oligonucleotides 408 and 404. The exon 15 and 16 sequence was amplified by PCR using oligonucleotides 407 and 401. The two PCR products were purified and then reamplified by overlapping PCR using oligonucleotides 408 and 401. The final products were digested with EcoRI and XhoI and then ligated into the pcDNA3-SP-G1 vector.

For the P5 construct, the GAG consensus sequence was amplified as for P4. Exon 15 was amplified by PCR using oligonucleotides 405 and 406. The GAG PCR and exon 15 PCR products were purified and then reamplified by overlapping PCR using oligonucleotides 405 and 401. The two PCR products were purified and then reamplified by overlapping PCR using oligonucleotides 408 and 402. The final products were digested with EcoRI and XhoI and then ligated into the pcDNA3-SP-G1 vector.

For the P6 construct, the GAG consensus sequence was amplified by PCR using oligonucleotides 408 and 406. Exon 16 sequence was amplified by PCR using oligonucleotides 407 and 401. The two PCR products were purified and then reamplified by overlapping PCR using oligonucleotides 408 and 401. The final PCR products were digested with EcoRI and XhoI and ligated into the pcDNA3-SP-G1 vector.

All PCR products were directly cloned into pCR-Script vector (Stratagene) following the vendor’s instructions and then digested with suitable enzymes prior to ligating them into a pcDNA3 vector. All constructs were separately transformed into E. coli strain TOP10F (Invitrogen) and amplified; the plasmid DNA was purified by Qiafilter Maxiprep (Qiagen) and sequenced by a university facility on a fee for service basis. Only constructs showing sequence identity to the desired gene sequences were used for transfection; any mutant constructs were discarded.

Cell Culture and Transfection—Chinese hamster ovary (CHO) cells were grown in minimal essential medium culture medium (Life Technologies, Inc.) supplemented with 5% fetal bovine serum and antibiotics (Life Technologies, Inc.; 100 units/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml amphotericin) at 37 °C in humidified air with 5% CO2. 1.8 million cells were seeded per 100-mm culture dish and cultured for 36 h prior to transfection. 15 μg of DNA, 52.5 μl of LipofectAMINE reagent (Life Technologies, Inc.) were each added to 500 μl of Opti-MEM (Life Technologies, Inc.) and mixed together. A 5-μl dish of cells was twice washed with Opti-MEM and then cultured with 5 μl of Opti-MEM. The DNA/LipofectAMINE mixture was then added to the cells, which were incubated for 6 h.

In Situ Cross-linking—Labeled cells were washed twice with ice-cold 130 mM NaCl, 20 mM Bicine, pH 8.0, lysed in ice-cold detergent plus cross-linker (50 mM Bicine, pH 8.0, 150 mM NaCl, 5 mM KCl, 5 mM MgCl2, 0.2% digitonin with 1.5 mM DTSSP, Pierce); and incubated on ice for 30 min. Cross-linker was inactivated with 10 mM glycine for 10 min on ice. The lysates were processed by a chelation chromatography method, described below. The cross-linked samples were electrophoresed in SDS-polyacrylamide gel electrophoresis in the presence of dithiothreitol, which cleaves an internal disulfide bond in DTSSP, releasing cross-linked proteins from each other.

Radioactive Labeling and Chelation Chromatography—After transfection, the medium was aspirated. For 35S labeling, 5 ml of fresh Opti-MEM and 250 μl of [35S]Na2SO4 (NEN Life Science Products) were added to the cells, which were incubated for 16 h. For [35S]methionine labeling, 5 ml of methionine- and cysteine-free Opti-MEM medium and 125 μl of [35S]-protein labeling mix (Amersham Pharmacia Biotech) were added to the cells, which were incubated for 15 h. The medium was collected, and protease inhibitors were added to final concentrations of 1 mM PMSF, 2 mg/ml leupeptin, 0.4 mg/ml antipain, 2 mg/ml benzamidine, 2 mg/ml aprotinin, 1 mg/ml chymostatin, 1 mg/ml pepstatin. Debris was removed by centrifuging at 1000 rpm for 1 min. 900 μl of medium was adjusted to 0.5% Triton X-100 concentration and added to 50 μl of nickel resin (Qiagen) for 1 h. Resin had been prepared by equilibration with 0.1 M NaCl, 44 mM NaHCO3, 1 mM imidazole, 0.5% Triton X-100. Sample-containing resin was spun at 14,000 × g for 15 s, and the liquid was decanted. Washes were performed with buffer A (10 mM HEPES, 2 mM NaCl, 10% glycerol, 0.1 mM PMSF, 2 mM imidazole, 0.5% Triton X-100, 1 mM MgCl2, 0.2% digitonin with 1.5 mM DTSSP, Pierce); and incubated on ice for 30 min. Cross-linker was inactivated with 10 mM glycine for 10 min on ice. The lysates were processed by a chelation chromatography method, described below. The cross-linked samples were electrophoresed in SDS-polyacrylamide gel electrophoresis in the presence of dithiothreitol, which cleaves an internal disulfide bond in DTSSP, releasing cross-linked proteins from each other.

Radioactivity Quantitation—Samples were collected at various times after adding radioactive isotope and electrophoresed. Radioactive protein bands were detected using an Instant Imager (Packard) and then dried and exposed to x-ray film.

Immunoprecipitation—Cell lysates or spent media were incubated with antibody to Hsp25 (Stressgen) and protein A beads (Pierce) on a rotating table at ice temperature for 5 h, following which the beads were harvested by centrifugation and serially washed with 50 mM Tris-HCl, pH 7.5, containing 1 mM NaCl and 1% Triton X-100 to elute nonspecific proteins. Then antibody-bound proteins were released by boiling the beads in sample buffer prior to SDS-polyacrylamide gel electrophoresis. SDS-Polyacrylamide Gel Electrophoresis and Autoradiography—The eluates or digested products were electrophoresed in a 5–15% gradient SDS-polyacrylamide gel electrophoresis gel, which was passed through an Instant Imager (Packard) and then dried and exposed to x-ray film.
RESULTS

This study was designed to further analyze how domain G3 governs the progression of aggrecan core protein through the intracellular routing pathway from the site of core protein synthesis in the ER to aggrecan's release at the cell surface. Various constructs were produced (Figs. 1 and 2) and individually transfected into CHO cells. Fig. 3 shows that the construct synthesized as a "miniaggrecan" core protein containing G1, GAG consensus sites and G3 was secreted at a rate intermediate between analogous proteins containing G1 or G3 alone. As described previously (9), the small amount of secreted G1 protein did not contain detectable GAG chains (data not shown), whereas secreted G3 protein or just GAG consensus sequences alone become neoproteoglycans (Fig. 3).

Globular proteins destined for secretion or targeted to the plasma membrane generally interact as nascent chains with ER intraluminal molecular chaperones; G1 and G3 domains would be expected to behave in a similar fashion. Pilot experiments showed that the molecular chaperone, Hsp25 (10, 11), appeared to interact with miniaggrecan core protein, and these observations were expanded. Fig. 4 shows that Hsp25 is present in CHO cells transfected with an empty vector or with a vector containing G1 or G3 constructs. Fig. 5a demonstrates that intracellular Hsp25 can be cross-linked to G3 protein but not to G1 protein; Fig. 5b shows that Hsp25 does not appear in the spent media in these experiments. As noted under “Experimental Procedures,” electrophoresis of the cross-linked proteins in the presence of dithiothreitol cleaves an internal disulfide bond in the DTSSP cross-link, liberating cross-linked proteins, in this case Hsp25 and G3.

The molecular positions of the G1 domain and the G3 domain are invariant throughout the members of the aggrecan family; G1 is always N-terminal to G3, implying that the locations of these globular domains are significant. Suitable constructs were transfected to test this hypothesis. Fig. 6 shows that locating G1 on the opposite flank of GAG consensus sites, in a construct termed RG1, reduces secretion compared with G1 construct; the small amounts of G1 and RG1 proteins in the media are not decorated with GAG chains (data not shown). Fig. 7 shows, in contrast, that locating G3 on the opposite flank of GAG consensus sites, in a construct termed RG3, does not affect the secretion rate and that G3 and RG3 proteins each become neoproteoglycans. It is noteworthy that, without chemical cross-linking, secreted RG3 protein is bound to Hsp25 (Fig. 7a) and that these two proteins remain firmly bound during chelation chromatography plus stringent washes followed by immunoprecipitation of the chromatographic eluate plus stringent washes (Fig. 8).

Exon-deleted constructs were used to determine which part(s) of domain G3 might underlie its ability to enhance...
intracellular routing and secretion of aggrecan-related proteins. Miniaggrecan core proteins lacking G3's epidermal growth factor domain (exon 13) or its sushi domain (exons 17–18) were fully functional (data not shown); only the C-lectin domain (exons 14–16) is needed for effective GAG chain O-glycosylation (Fig. 9) and miniaggrecan secretion (Fig. 10). Within the C-lectin domain, exon 15 accounts for virtually all of G3's properties (Figs. 9 and 10); exons 14 and 16 contribute little to exon 15's properties, but each, by itself, suppresses those properties. Interestingly, two separate proteins originate from the exon 15 construct (Fig. 10, lane 7), and two different populations of neoproteoglycans seem to arise from those proteins (Fig. 9, lane 7).

**DISCUSSION**

Secreted proteins with multiple globular domains, e.g. members of the aggrecan family, would be expected to undergo chaperone-mediated surveillance in the ER lumen. It is difficult to visualize, however, how multidomain surveillance might be orchestrated and regulated for an individual molecule. Various possibilities include separate chaperones for each globular domain (1) and/or “intramolecular” chaperones (12).

The experimental results in this study confirm earlier work that proteins bearing domain G3 are secreted more rapidly than those containing domain G1 (9). Interestingly, a G1 plus G3-containing protein is secreted at a rate intermediate between a protein bearing G1 alone and one bearing G3 alone. These observations suggest that G3 facilitates secretion of G1 and, consequently, of aggrecan core protein itself.

**Aggrecan Globular Domains—** Members of the aggrecan family contain G1 at the N-terminal flank of the GAG consensus sites and G3 at their C-terminal flank. Reversing these orientations decreases the secretory rate of G1 protein but does not

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**Fig. 2.** Diagram of constructs designed to test the putative role of aggrecan's C-lectin domain in neoproteoglycan formation and secretion. Various sequences are identified in Fig. 1. The complement regulatory protein-like domain is synonymous with the short consensus repeat and sushi domains. The exons coding for their respective domains are depicted. All constructs were sequenced to determine fidelity prior to insertion into a eukaryotic expression vector.

**Fig. 3.** G1, G3, and G1 + G3 (miniaggrecan) protein secretion, labeled by [35S]methionine and secretion of neoproteoglycans, labeled by [35S]Na2SO4, a, [35S]methionine label; spent media; autoradiography of empty vector proteins (lane 1), G1 protein (lane 2), G3 protein (lane 3), and G1 + G3 protein (lane 4). The arrows depict the locations of the secreted proteins. b, cell lysates (same samples as in panel a). c, percentage of each protein secreted by cells. Cells were labeled for 16 h in these experiments. d, [35S]Na2SO4 label; spent media; autoradiography of empty vector proteins (lane 1), SP-GAG5-G3-HIS6 protein (lane 2), and SP-GAG5-HIS6 protein (lane 3).

**Fig. 4.** The presence of Hsp25 in cell lysates of CHO cells, labeled by [35S]methionine. Cell lysates were immunoprecipitated with nonspecific antibody (Ab, –) or anti-Hsp25 antibody (Ab, +). Empty vector transfection (lanes 1 and 2), G1 construct transfection (lanes 3 and 4), and G3 construct transfection (lanes 5 and 6) are shown. Cells were labeled for 6 h in these experiments.
affect the G3 protein secretory rate, suggesting that both individual globular conformation and globular location are important determinants contributing to intracellular passage of G1/G3-containing proteins.

The three-dimensional structure of rat mannose-binding protein has been deciphered from its crystalline form (13); the highly conserved secondary structure of its C-lectin domain is prevalent in a wide variety of otherwise unrelated proteins (14). Using the atomic coordinates of rat mannose-binding protein, molecular modeling has yielded homologous secondary structure (Figs. 11 and 12) of the C-lectin domain of mouse IgE cell surface receptor (15); this model appears prototypical of the secondary structure of all the related C-lectin domains (16) and is reflected in the primary structure as well. For example, comparison of the amino acid sequences of mouse IgE receptor C-lectin domain and chicken aggrecan C-lectin domain shows 38% conservation (Fig. 13); the other proteins in this superfamily generally show 20–40% conservation of secondary structure, compared with the mouse IgE receptor C-lectin domain (14). Recent expansion and further refinement of these analyses shows that a superfamily of 131 secretory or cell surface
Cells were labeled for hi in these experiments.

**FIG. 9.** Secretion of neoproteoglycans, labeled by $[{\text{35}}\text{S}]\text{Na}_2\text{SO}_4$. Secretion of neoproteoglycans containing exon-based deletions; autoradiography of empty vector proteins (lane 1), SP-G1-GAG-G3-His$_6$, or miniaggrecan protein (lane 2) and proteins P1 (lane 3), P2 (lane 4), P3 (lane 5), P4 (lane 6), P5 (lane 7), and P6 (lane 8). One bracket depicts the neoproteoglycan based on miniaggrecan protein while the other bracket depicts the neoproteoglycan based on protein P1 or P5.

Miniaggrecan protein and exon-deleted miniaggrecan secretion, labeled by $[{\text{35}}\text{S}]$methionine. a, spent media; autoradiography of empty vector proteins (lane 1), miniaggrecan protein (lane 2), P1 protein (lane 3), P2 protein (lane 4), P3 protein (lane 5), P4 protein (lane 6), P5 protein (lane 7), and P6 protein (lane 8). b, cell lysates (same samples as in panel a). c, percentage of each protein secreted by cells. Cells were labeled for 6 h in these experiments.

**FIG. 10.** Space-filling representation of the C-lectin domain of mouse IgE receptor. This figure was created in the same way as Fig. 11 except that the space-filling mode of representation was substituted for the ribbon mode.

**FIG. 11.** Ribbon representation of the C-lectin domain of mouse IgE receptor, a prototypical member of a superfamily of 131 different proteins, including chicken aggrecan core protein. This conceptual model (15) was based on the known structure of rat mannose-binding protein, a member of the superfamily. The color-coded sections represent the equivalent regions encoded by chicken aggrecan exons. The graphics programs Swiss-PdbViewer (available on the World Wide Web at expasy.hcuge.ch/spdbv/mainpage.html) and POV-Ray (available at www povray.org/) were used to create this figure.

proteins has a consensus sequence of 136 residues, with 32% of them being conserved (17); one of the two calcium binding sites is partially encoded by exon 15, and the remainder is encoded by exon 16 in the avian domain.

The C-lectin domain of G3 has recently been shown to bind tenascin R via calcium-mediated protein-protein interaction (6). Assuming that it is exon 15 protein that binds tenascin R, it may also be the site that binds Hsp25. The environment of the ER lumen provides ideal circumstances for such interactions because it has high concentrations of calcium, molecular chaperones, and nascent proteins (18, 19). The protein encoded by exon 15 has two prominent lobules, which we speculate may, in their nascent state, be the actual binding site for Hsp25. Once the lobules are formed, Hsp25 may dissociate, enabling the lobules to interact elsewhere. As proposed below, once Hsp25 is released, the G3 lobules may bind to the nascent G1 region of core proteins, acting as an "intramolecular" chaperone. The failure of Hsp25 to dissociate from RG3 may potentially provide insight into the recognition mechanisms between that molecular chaperone and nascent aggrecan core proteins. Conservation of the C-lectin domain structure and its widespread occurrence as a common unit of many exported and cell surface proteins imply that it is an important recognition signal for ER quality control mechanisms. In some cases, the C-lectin domain structure may be expendable once the parent protein reaches its final destination. This may, in fact, be the case for extracellular aggrecan, which progressively loses its entire G3 domain (20).

**Molecular Chaperone Hsp25**—The small heat shock proteins, including Hsp25 (11, 21), are well conserved in prokaryotes, animals, and plants, sharing internal domains of about 35 amino acids within the carboxyl-terminal half of the proteins (22, 23). To date, plant cells show the most diverse intracellular distribution of small heat shock proteins, which are distributed in cytoplasmic classes I and II, in chloroplast class III, and in the ER lumen as class IV (24). This latter class was found most recently, and the authors suggest that it may have gone unoberved in other eukaryotes. Although three alternatively spliced Hsp25 isoforms are seen in cultured marine osteoblasts and their relative expression is controlled by two separate promoter elements, none of these isoforms has an N-terminal signal sequence (25).

One possibility is that cytosolic Hsp25 may bind to nascent G3 during a translocational pause; such pauses have been linked to potential nascent chain interactions with cytosolic components (26, 27). Pause sites have been mapped in nascent apolipoprotein B-100 but do not show linear consensus sequences (27); the authors invoke topogenic consensus sequences, conceptually analogous to chaperone topogenic recognition sequences.
G3, but not G1, interacts with molecular chaperone Hsp25; such specificity is consistent with chaperone quality control mechanisms in the ER lumen and trafficking of properly folded proteins. This point is emphasized by the unexpected observation that Hsp25 and RG3 protein remain associated in a complex that is released from the cell, suggesting that intracellular Hsp25 fails to dissociate once RG3 has achieved proper conformation. It is also noteworthy that 1) persistent binding of Hsp25 does not prevent GAG chain addition to consensus sites in RG3 protein and 2) the precise molecular location of the G3 domain in a protein has a profound effect on Hsp25 interactions.

Scheme for Hsp25-G3 Interactions—Fig. 14 illustrates one possible scheme that is consistent with current results. Nascent domain G3 initially binds cytosolic Hsp25 during a translocational pause, bringing Hsp25 into the ER lumen and preventing G3 aggregation, thereby enabling intraluminal G3 to adopt its correct conformation. Intraluminal G3 then dissociates from properly folded G3, which in turn serves as an intramolecular chaperone and binds to G1, enabling G1 to progressively fold into proper conformation. G3 could, of course, interact with G1 in the same nascent molecule and/or with G1 in adjacent nascent molecules, similar to schemes proposed for other intramolecular chaperones (28). Meanwhile, released Hsp25 is probably recycled to the nucleus, consistent with its intranuclear localization in stressed cells (29); it is generally recognized that stress reactions simply emphasize preexisting physiological pathways.

Although G3 enhances G1 routing through the cell, G1 appears to be immature when it leaves the cell, denoted by its initial weak avidity for hyaluronic acid. That avidity progressively increases once aggrecan is in the extracellular matrix (30), apparently due to G1 achieving correct disulfide bond arrangements (31). Thus, while G3 enables G1 to traverse the intracellular quality control pathway, such passage is insufficient for G1 to perfect its final conformation. Extracellular G1 gradually matures, eventually fulfilling its major role in participating in an extended network with hyaluronic acid and link proteins. Simultaneously, extracellular aggrecan progressively loses its G3 domain (20), consistent with a predominantly intracellular role.

Ramiifications of Hsp25 Expression and Regulation—Recent research has implicated Hsp25 as being protective against oxidative stress and subsequent apoptosis (32). Constitutive expression of human Hsp25 in murine cells blocks receptor-initiated cell death (33); Hsp25 levels are developmentally regulated and have been linked to cell growth and differentiation (34). Hsp25 transient expression is critical for preventing differentiating ES cells from undergoing apoptosis, by a switch that may be redox-regulated (35). Redox levels in the ER lumen are maintained by inflow of cysteine and glutathione (36); enhanced synthesis of secretory and membrane proteins stimulates such thiol influx to counteract the vectorial import of reduced cysteine residues that enter the ER lumen as part of nascent proteins. The precise relationships among Hsp25 regulation, cellular redox levels, and apoptosis have not been elucidated.

Hsp25 is constitutively expressed in a wide variety of vertebrate tissues (37). Of particular interest is the localization of this molecular chaperone to the upper part of the hypertrophic zone of the rat tibial growth plate (38). This region is a site of active synthesis of cartilage matrix components including aggrecan. Growth plate hypertrophic chondrocytes progressively move toward bone, successively becoming apoptotic (39–41) Thus, down-regulation of Hsp25 in hypertrophic chondrocytes correlates with diminished synthesis of aggrecan and switching to an apoptotic pathway. Chondrocytes in postnatal cartilage, a relatively anoxic environment, may be especially sensitive to such a redox-regulated switch. This point is reflected by chondrocytes in culture, since thiol levels in the culture medium are found to be critical in modulating events linked to chondrocyte maturation and cartilage matrix synthesis (42).

In summary, molecular chaperone Hsp25 has been implicated in the intracellular trafficking of aggrecan core protein,
and, most likely, Hsp25 participates in a quality control system that determines whether the core protein will leave the ER lumen to enter the Golgi stacks. Hsp25 interacts with the C-lectin motif of domain G3; strikingly, Hsp25 occurs throughout eukaryotes, and, concomitantly, the C-lectin motif is seen in an extensive variety of cell surface and secreted eukaryotic proteins, implying that an evolutionary partnership between the molecular chaperone and the protein motif has been very well conserved.

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REFERENCES

1. Hammond, C., and Helenius, A. (1995) Curr. Opin. Cell Biol. 7, 523–529
2. Wei, J., and Hendershot, L. (1996) Exper. Suppl. (Basel) 77, 41–55
3. Ruoslahti, E. (1996) Glycobiology 6, 489–492
4. Hardingham, T. E., Fosang, A. J., and Drickamer, K. (1992) Nature 358, 505–511
5. Engel, K., Knauf, U., and Gaestel, M. (1991) Biomed. Biochim. Acta 50, 1065–1071
6. Schinde, U., Li, Y., Chatterjee, S., and Inouye, M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 6924–6928
7. Weiss, W. L., Drickamer, K., and Hendrickson, W. A. (1992) Nature 360, 127–134
8. Schneider, R., de Daruvar, A., and Sander, C. (1997) Nucleic Acids Res. 25, 226–230
9. Luo, W., Kuwada, T., Chandrasekaran, L., Zheng, J., and Tanzer, M. L. (1996) J. Biol. Chem. 271, 16447–16450
10. Cooper, L. F., and Uoshima, K. (1994) J. Biol. Chem. 269, 7869–7873
11. Shinde, U., and Inouye, M. (1995) J. Biol. Chem. 270, 1647–1652
12. Brissett, N. C., and Perkins, S. J. (1998) Biochim. Biophys. Acta 1394, 226–230
13. Padlan, E. A., and Helm, B. A. (1993) Receptor 3, 325–341
14. Drickamer, K., and Taylor, M. E. (1993) Annu. Rev. Cell Biol. 9, 237–264
15. Golovina, V. A., and Blaustein, M. P. (1997) Science 275, 1643–1648
16. Gething, M. (1996) Curr. Biol. 6, 1573–1576
17. Teixeira, C. C., Shapiro, I. M., and Hatori, M. (1996) J. Bone Miner. Res. 11, 1643–1650
18. Sandy, J. D., O’Neill, J. R., and Ratcliffe, L. C. (1989) Biochem. J. 258, 875–880
19. Sandy, J. D., and Plaa, A. H. (1989) Arch. Biochem. Biophys. 271, 300–314
20. Mehlen, P., Kretz-Remy, C., Briolay, J., Fostan, P., Mirault, M. E., and Arrigo, A. P. (1995) Biochem. J. 312, 367–375
21. Mehlen, P., Schulze-Osthoff, K., and Arrigo, A.-P. (1996) J. Biol. Chem. 271, 16510–16514
22. Arrigo, A. P. (1995) Neuropathol. Appl. Neurobiol. 21, 488–491
23. Mehlen, P., Mehlen, A., Godet, J., and Arrigo, A.-P. (1997) J. Biol. Chem. 272, 31657–31665
24. Sandy, J. D., and Plaa, A. H. (1989) Arch. Biochem. Biophys. 271, 300–314
25. Mehlen, P., Meheen, A., Godet, J., and Arrigo, A.-P. (1995) Biochem. J. 272, 31657–31665
26. Carelli, S., Ceriotti, A., Cabilbo, A., Fassina, G., Ruvo, M., and Sita, R. (1997) Science 277, 1681–1684
27. Ciocca, D. R., Oesterrech, S., Channeness, G. C., McGuire, W. L., and Faqua, S. A. (1993) J. Natl. Cancer Inst. 85, 1558–1570
28. Vannuyelen, N., Evard, L., and Dourou, N. (1997) Anat. Embryol. 195, 359–362
29. Hatori, M., Klatte, K. J., Teixeira, C. C., and Shapiro, I. M. (1995) J. Bone Miner. Res. 10, 1960–1968
30. Zenmyo, M., Komiyama, S., Kawabata, R., Sasaguri, Y., Inoue, A., and Morimatsu, M. (1996) J. Pathol. 180, 430–433
31. Teixeira, C. C., Shapiro, I. M., Hatori, M., Rajpurohit, R., and Koch, C. (1996) J. Bone Miner. Res. 11, 1643–1656
32. Silverman, M., and Hwang, E. Y. (1995) Trends Biochem. Sci. 20, 205–211