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Two Alanines Juxtaposed to Aggrecan's G1 Domain Alter its Intracellular Localization

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Abstract Nascent proteins translated and processed in the endoplasmic reticulum (ER) sometimes contain intrinsic signals for ER retention or ER retrieval. These signals are usually a few amino acids in length, and if alanine modifications are made within these sequences, normal transit patterns of the nascent protein frequently change. The purpose of this study was to determine whether two alanines juxtaposed to the first globular domain of aggrecan's core protein affect its transit in Chinese hamster ovary (CHO) cells. Results show that two alanines juxtaposed to the first globular domain (G1AA) minimized secretion of the protein. However, transgenic proteins with juxtaposed glutamate–phenylalanine (G1EF) or no additional amino acids (G1) were still secreted. GFP-tagged G1AA localized in the lumen of the ER but not in the Golgi. In contrast, a portion of GFP-tagged G1EF and G1 did appear in the Golgi compartment. More importantly, unique and striking accumulations of G1EF and G1 transgenic proteins were seen in large dilated regions of the ER cisternae, reminiscent of accumulations seen in α1-antitrypsin deficiency disease. G1AA transgenic proteins did not form these vesicles but were diffusely distributed throughout the ER lumen. These results indicate that just two juxtaposed alanines can profoundly affect a large globular protein's intracellular localization. J. Cell. Biochem. 90: 592–607, 2003.

Key words: proteoglycan; endoplasmic reticulum; intracellular trafficking; ER accumulations

Secreted proteins are translated in the endoplasmic reticulum (ER) and modified in the Golgi compartment before transport to the extracellular space. However, certain proteins are permanent residents of the ER and are either retained in or recycled there as a result of specific amino acid sequences that act as the signals for retention or recycling. The most studied sequence is KDEL, which signals retrieval of proteins from the Golgi to the ER via a specific receptor protein. Specifically, when KDEL is at the carboxyl terminus of a protein, that protein is constantly recycled back to the ER from the Golgi [Munro and Pelham, 1987] while mutating the sequence to KDAS promotes protein secretion instead of recycling [Liaudet et al., 1994]. A similar example is the dilysine sequence, KKXX, that mediates transmembrane protein recycling to the ER from the Golgi [Jackson et al., 1990]. However, substituting alanines (KKAA) for XX results in ER retention [Andersson et al., 1999]. The ER export signal, FCYENE, was discovered in the inwardly rectifying potassium channel [Ma et al., 2001] and is required for proper ER export and localization of the channel to the plasma membrane. But when that sequence is mutated to FCYANA, the mutant channel is retained in the ER. Finally, the di-acidic signal, DXE, is specific for ER to Golgi anterograde transport, while substitution of alanines for D and E (AXA) localizes the mutant protein to the ER [Nishimura and Balch, 1997]. Interestingly, whenever alanines were substituted in these signaling sequences, the protein's fate changed so that it was either retained in the ER (as in the case of KKAA, FCYANA, and AXA) or targeted for secretion (as

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in the case of KDAS). This evidence indicates that, in certain cases, alanines may play an active role in altering a protein's intracellular trafficking pathway rather than a passive role as a place holder.

We describe, in this report, studies on intracellular localization after altering amino acids in an aggrecan core protein domain. Aggrecan is a large aggregating proteoglycan essential to functional cartilage where it binds type II collagen, link protein, and hyaluronan. It comprises a core protein with three globular domains (G1, G2, and G3) and a linear, interglobular domain (GAG) with attached glycosaminoglycan side chains between G2 and G3 (Fig. 1A) [Schwartz et al., 1999; Luo et al., 2000; Schwartz, 2000]. Aggrecan core protein is translated and inserted into the lumen of the ER where the globular domains are properly folded with the help of chaperones, Hsp25 and Hsp70 [Schwartz et al., 1999; Luo et al., 2000]. The correctly folded core protein then transits to the Golgi complex where glycosaminoglycan chains, predominantly keratan sulfate and chondroitin sulfate, are added to the core protein [Luo et al., 2000; Vertel and Ratcliff, 2000]. Post-Golgi secretory vesicles carry the proteoglycan to the cell surface where it is secreted into the extracellular milieu. Previous work done in this laboratory identified the specific domains of aggrecan core protein that are needed for efficient secretion. Generally, the third globular domain (G3) and specifically, exon 15 are necessary for aggrecan's efficient secretion [Zheng et al., 1998]. Continuing those studies, we refocused on the G1 domain and designed new constructs that placed a glutamate (E) and phenylalanine (F) next to the G1 domain (creating a restriction site that could serve as a cut site). Keeping in mind the trafficking fate of KKAA, FCYANA, and AXA from previous studies, we placed two alanines in the same site as the EF in another construct. When the two transgenes were tested for their transfection efficiency and secretory behavior, the transgenic protein that contained two alanines was not secreted while the EF-containing protein was secreted. In order to clarify the nature of this observation, we studied the secretion and intracellular localization of the transgenic proteins in Chinese hamster ovary (CHO) cells.

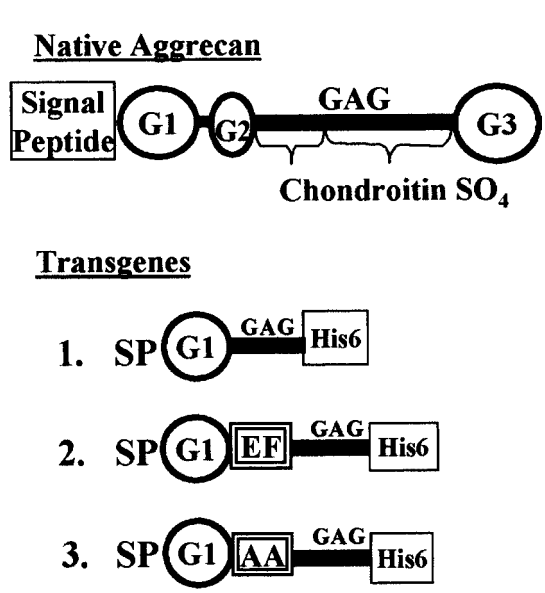


Fig. 1. Diagrams of native aggrecan and the basic transgenic protein constructs. **A**: Native aggrecan consists of signal peptide (SP) that targets nascent peptides to the endoplasmic reticulum (ER); G1, G2, and G3 that are aggrecan's first, second, and third globular domains, respectively; and GAG, the consensus sequence that contains the sites of keratan sulfate and chondroitin sulfate attachment. **B**: The basic transgenic protein (1) has the SP followed by G1 domain, a 159 amino acid portion of native GAG and a histidine hexapeptide tag (His6). Either glutamate–phenylalanine (2) or two alanines (3) were juxtaposed C-terminal to G1. The GAG sequence was identical in all of the constructs. All constructs were sequenced prior to use to verify fidelity.

MATERIALS AND METHODS

Transgene Construction

All transgene constructs contain the endogenous avian aggrecan signal peptide (SP) at the 5′ end, plus or minus a six-histidine (His6) tag, plus or minus a pEGFP-N3 cassette at the 3′ end (Fig. 1B). The detailed methods for SP, G1, and GAG construction were published previously [Zheng et al., 1998]. The His6 tag was retained in all transgenes for consistency even though we relied on GFP for cellular visualization of transgenic product. Control studies (see "Results") showed that GFP did not alter the intracellular location of the transgenic proteins. Oligonucleotides used for the transgenic constructs are given in Table I. Briefly, G1EF was amplified by PCR using oligonucleotides 408 and 569. The PCR products were ligated to predigested pPCR-Script Amp SK(+) cloning vector (Stratagene, LaJolla, CA) for transforming XL10-Gold Kan ultracompetent cells (Stratagene). *E. coli* colonies were screened, suitable inserts were ligated into the expression vector, $pcDNA_3$ (Invitrogen, Carlsbad, CA), and used to transform Top 10'F cells (Invitrogen).

TABLE I. Oligonucleotides Used to Make Transgenic Constructs

Oligo	Oligonucleotide sequence	Restriction sites
1-139	CAGCCAGGATCCATGACCACTCTACTACTAG[a]	*Bam*H1
2-139	ATATATATATGCGGCCGCCCCTGGGACCAGAGCCTCGAA	*Not*1
3-139	CATATAGCGGCCGCCTTCCCTGAAATTAGCGTAG	*Not*1
4-139	GCGCGCTCTAGACTAATGATGATGATGATGATG	*Xba*1
408	GCGATGGAATTCGCCTTCCCTGAAATTAGC	*Eco*RI
569	GCGCTCGAGCTAATGATGATGATGATGAT-GTTCTGTCGTGGGTCCCAG	*Xba*1
601	GAGCTCGAGATGACCACTCTACTACTAGTG	*Xho*1
602	CGCCGAGGATCCATGATGATGATGATGATG	*Bam*H1
801	TATAGGATCCCCCTGGGACCAGA	*Bam*H1
802	TAGGATCCGAATTCCCCTGGGACCAGA	*Bam*H1
803	TATATATAGGATCCGGCCGCCCCTGGGACCA	*Bam*H1

[a]All sequences are given 5′ to 3′.

G1AA was amplified by PCR using oligonucleotide 1-139 (*Bam*H1 site) plus 2-139 (*Not*1 site). AA-GAG-His6 was amplified by PCR using oligonucleotide 3-139 (*Not*1 site) plus 4-139 (*Xba*1 site), which contains His6 and a stop codon. The PCR products from the two different reactions were ligated separately to the pPCR-Script Amp SK(+) vector as above. After transformation, a full length G1AA was obtained. The GFP-containing transgenes were constructed as above except there were no stop sites in the first purified product. After removing the insert from the cloning vector, it was ligated to the corresponding sites of the pEGFP-N3 vector (Clontech, Palo Alto, CA).

The plasmid DNA from all constructs was purified by Qiafilter Maxiprep (Qiagen, Valencia, CA) 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.

Cell Culture and Transfection

CHO-K1 cells (American Type Culture Collection, Rockville, MD) were maintained at 37°C in humidified air with 5% CO_2 in complete medium consisting of minimum essential medium (MEM) (Mediatech, Inc., Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 50 U/ml penicillin, and 50 μg/ml streptomycin (all from Life Technologies, Gaithersburg, MD). A few parallel studies were done using C28/I2 chondrocytes, an immortalized human juvenile costal cartilage cell line (generous gift of Dr. Mary Goldring) [Goldring and Berenbaum, 1999]. These cells were maintained as above except the medium used was 1:1 of Ham's F-12 and MEM supplemented as described.

All cells were cultured to subconfluence either in 100 mm^2 dishes for biochemical studies or on 22-mm square glass coverslips for immunofluorescence. CHO cells were transfected as previously described with minor modifications [Zheng et al., 1998]. Briefly, the construct DNA, PLUS reagent, lipofectAMINE reagent (Life Technologies, Inc.) and Opti-MEM (Life Technologies, Inc.) were combined. Concurrently, a dish of cells was washed twice with Opti-MEM. The DNA–LipofectAMINE-PLUS mixture was added to the cells, which were incubated for 2 h. The transfection medium was then replaced with complete medium (zero time point), incubated for the appropriate time periods as stated in the figure legends, and then used for immunofluorescence or biochemical studies.

Studies using nocodozole and Brefeldin A (BFA) were done as follows: CHO cells were transfected as usual. At the same time that the transfection medium was changed to complete medium, either 10 μg/ml of nocodozole (Calbiochem, San Diego, CA), 5 μg/ml of BFA (Calbiochem), or vehicle was added.

Radioactive Labeling and Chelation Chromatography for Biochemical Studies

After transfection, the medium was aspirated and [^{35}S]methionine labeling was done as previously described [Luo et al., 1996; Zheng et al., 1998]. To each 100 mm^2 culture dish, 1 mCi of ^{35}S-protein labeling mix (specific activity: >1,000 mCi/mmol, Amersham Pharmacia Biotech, Inc., Piscataway, NJ) was added. After timed incubations with the radiolabeled medium, the spent medium was collected, clarified, and protease inhibitors were added to final concentrations of 1 mM PMSF, 2 μg/ml leupeptin, 0.4 μg/ml antipain, 2 μg/ml benzamidine, 2 μg/ml aprotinin, 1 μg/ml chymostatin, 1 μg/ml pepstatin. In order to select and isolate only His6-containing transgenic proteins, nickel resin chelation was done. To 900 μl of medium,

50 μl of nickel resin (adjusted to 0.5% Triton X-100) (Qiagen) was added with rotation for 1 h. Nickel resin was prepared by equilibration with 0.1 M NaCl, 44 mM $NaHCO_3$, 1 mM imidazole, 0.5% Triton X-100. After centrifuging at 14,000*g* for 15 s, resin was washed three times with buffer A (10 mM HEPES, 2 M NaCl, 10% glycerol, 0.1 mM PMSF, 2 mM imidazole, 0.5% Triton X-100, 6 M urea, 250 mM dithiothreitol, pH 8.0) for 30 min and then with buffer B (10 mM HEPES, 750 mM NaCl, 10% glycerol, 0.1 mM PMSF, 10 mM imidazole, 0.5% Triton X-100, 1 mg/ml bovine serum albumin, pH 8.0) for 30 min, buffer C (10 mM HEPES, 750 mM NaCl, 10% glycerol, 0.1 mM PMSF, 10 mM imidazole, pH 8.0) for 15 min and eluted with 10 mM HEPES, 750 mM NaCl, 10% glycerol, 100 mM EDTA, 0.1 mM PMSF, 250 mM imidazole, pH 8.0, for 1 h. An alternate method was used to isolate G3-containing transgenes from medium (Fig. 7F). After 18 h transfection, medium was collected and concentrated using Millipore Ultrafree®-15 Centrifugal Concentrators (Fisher Scientific, Suwanee, GA) with a 30 kDa molecular weight cutoff. Equivalent protein of the concentrated samples was separated using 12% SDS/PAGE, electrophoretically transferred to membrane, and probed with anti-His6 antibody (Amersham Pharmacia) followed by enhanced chemiluminescence detection (Amersham Pharmacia).

In order to purify and isolate His6-containing transgenic proteins from the cell lysates, the nickel resin chelation protocol was used. After the spent medium was removed, the cells were washed twice with Hanks' medium (Life Technologies, Inc.) and lysed with 1 ml of 0.5% Nonidet P-40 on ice for 10 min. The lysate was clarified and 200 μl of the lysate plus 700 μl of Opti-MEM were adjusted to 0.5% Triton X-100 concentration. The lysate was then processed for His6-containing proteins with nickel resin following the prior protocol.

For [^{35}S]sulfate labeling to identify neoproteoglycans, 5 ml of fresh sulfate-free Opti-MEM and 250 μl of sodium [^{35}S]sulfate (NEN Life Science Products, Boston, MA) (specific activity: 250–1,000 mCi/mmol) were added to the transfected cells, which were then incubated for 4, 8, or 12 h in the presence of the radioactivity [Zheng et al., 1998]. Spent medium was processed for His6-containing neoproteoglycans with nickel resin following the prior protocol.

Radioactivity Quantitation

After samples were collected and processed, equivalent aliquots of each were electrophoresed using 5–15% gradient SDS–polyacrylamide gels. After drying, the gels were exposed to X-ray film for 1–3 weeks. Radioactively labeled protein bands were photographed and quantitated using an Instant Imager (Packard Instruments, Meriden, CT). The optical density of each sample was corrected by subtracting the optical density of samples transfected with control (no insert) vectors. Optical density is expressed as non-specific units. Percent secreted is expressed as the amount of units in the medium divided by the sum of the amount of units in the medium plus the amount of units in the cell lysate. Percent not secreted is expressed as the amount of units in the lysate divided by the sum of the amount of units in the cell lysate plus the amount of units in the medium.

Antibodies

Anti-calnexin carboxyl terminus polyclonal antibody was obtained from StressGen Biotechnologies Corp. (Victoria, BC, Canada) and was used to visualize the ER [Wada et al., 1991]. Dr. Kelley Moremen (University of Georgia) supplied the anti-mannosidase II antibody that was used to identify the Golgi compartment [Novikoff et al., 1983; Moremen and Robbins, 1991]. The antibodies to beta-COPI and COPII were purchased from Affinity BioReagents, Inc. (Golden, CO). The fluorescent secondary antibodies (Alexa 488 and Alexa 594) were from Molecular Probes, Inc. (Eugene, OR).

Immunofluorescent Labeling

Cells were grown on 22 mm^2 glass coverslips in 6-well plates. After transfection for 14 h, cells were washed three times with PBS. Cells were then fixed and permeabilized with ice cold methanol/acetone (3:7, v/v) for 10 min at −20° C, followed by washing five times with PBS. After blocking with PBS containing 10% goat serum (blocking buffer) for 1 h at room temperature, cells were washed with PBS, and then incubated with the primary antibody diluted in blocking buffer for 1 h at room temperature. After washing five times with PBS containing 0.1% Triton (PBS-T), cells were incubated with the secondary fluorescently-labeled antibody for 1 h at room temperature. Again, cells were washed five times with PBS-T. Coverslips

were mounted on slides using SlowFade antifade reagent (Molecular Probes) and viewed with conventional epifluorescence or confocal microscopy. A Leica DMR epifluorescent microscope (Empire Imaging Systems, Asbury, NJ) was used for conventional microscopy. Digital images were captured using a CoolSnap monochrome CCD camera (BioVision Technologies, Exton, PA) and RS Imaging software (Roper Scientific, Inc., Trenton, NJ). Confocal images were captured using a Zeiss LSM 510 inverted confocal microscope (Carl Zeiss, Inc., Thornwood, NY) mounted on an Axiovert 100 M and fitted with argon and helium/neon lasers. Merged images were collected as RGB images using LSM software.

Electron Microscopy

Transfected cells were grown to confluence in 100-mm culture dishes and the medium was replaced with fixative solution containing 3% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. After fixation for 1 h, the fixative solution was replaced with 1% paraformaldehyde in cacodylate buffer and the cells were stored at 4°C. The cells were carefully removed from the dishes with a Teflon scraper, centrifuged, and embedded in low-gelling agarose. The cell pellets were dehydrated in cold methanol and embedded in LR Gold resin at −20°C. Thin sections of the cell pellets were cut with a diamond knife and collected on formvar-coated nickel grids.

Immunogold labeling was carried out as previously described [Hand, 1995]. Briefly, the sections were treated with 1% BSA and 1% instant milk in PBS to block non-specific binding, then incubated with rabbit anti-GFP (Clontech) in 1% BSA and 5% normal goat serum in PBS overnight at 4°C. The sections were rinsed with PBS and then incubated with goat anti-rabbit IgG labeled with 10 nm diameter gold particles (Amersham Pharmacia) for 1 h at room temperature. After thorough rinsing with PBS and distilled water, the sections were stained with uranyl acetate and lead citrate, and examined in a Philips CM10 TEM at 60 kV.

RESULTS

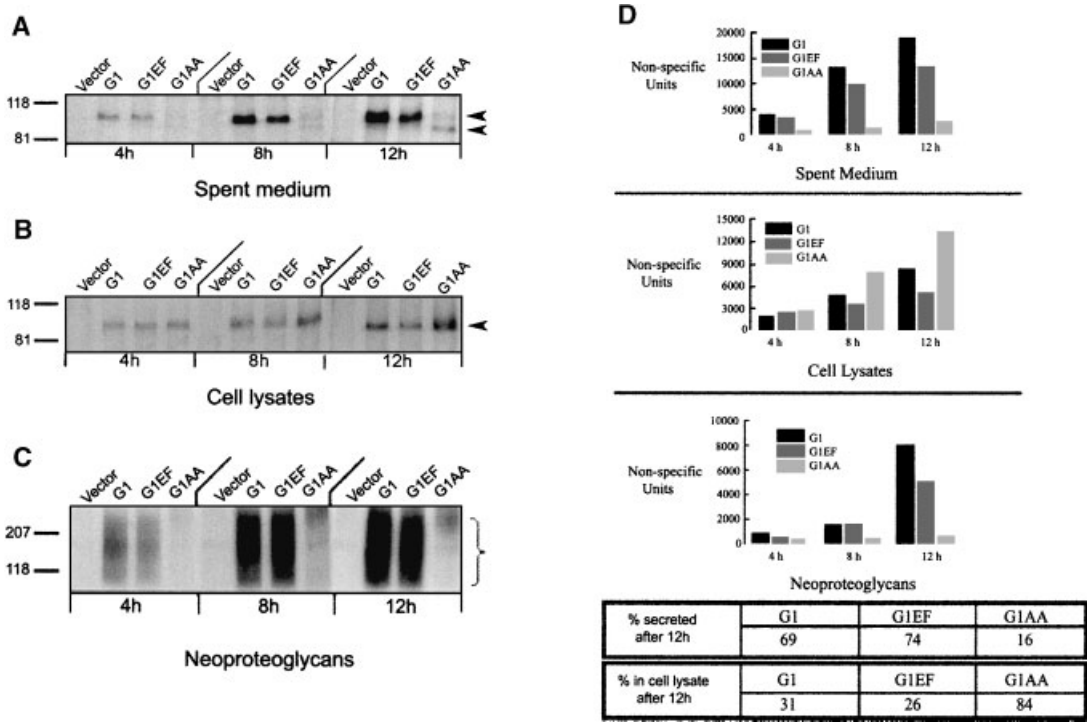
Construction of Aggrecan Transgenes

Aggrecan's core protein consists of a SP, three globular domains (G1, G2, and G3) and glycosaminoglycan consensus sites (GAG) (Fig. 1A). We constructed a series of transgenes to determine the effect of amino acid additions and modifications on the intracellular localization and trafficking of aggrecan's first globular domain (Fig. 1B). The first series of transgenes used a primary construct that has an initial SP, the first globular domain of the aggrecan core protein, a short GAG sequence taken from the C-terminal portion of full length aggrecan, a His6 tag, and a stop site. This transgene (G1) transits slowly through the ER and Golgi [Zheng et al., 1998] and was used as the basis for constructing the other transgenes. The genetic codons for two amino acids, either glutamate–phenylalanine (EF) or alanine–alanine (AA), were juxtaposed C-terminal to G1 (Fig. 1B) to yield G1EF and G1AA, respectively. The GFP-tagged transgenes, constructed by adding an EGFP cassette to the C-termini of the basic transgenes, were designated G1-GAG-GFP, G1EF-GAG-GFP, and G1AA-GAG-GFP.

Secretion of Aggrecan Transgenic Proteins

The amount of transgenic protein processed and secreted into the culture medium after transfection of CHO cells was measured. Secreted G1 and G1EF transgenic proteins were detected in the spent medium of CHO cells at 4, 8, and 12 h (Fig. 2A). The bands represent methionine-labeled, newly synthesized protein. Quantitative analyses of the autoradiographs (Fig. 2D, top panel) showed that over half of measured G1 and G1EF was already secreted into the spent medium at 4 h post-transfection while the remaining protein was still inside the cell (Fig. 2D, middle panel). Very little measured G1AA (12%) was in the medium at that time point. By 12 h, the majority of measured G1 and G1EF (70%) were secreted into the spent medium while measured G1AA was still barely detectable. This slight amount of G1AA was visible as a very faint lower molecular weight band (Fig. 2A) that most likely represented a very small amount of an extracellular degradation product that still contained the hexahistidine tag used to purify the protein. There were no other protein bands in this lane indicating that the majority of this transgenic protein was not secreted. In fact, the percent of measured G1AA isolated from cell lysate at 12 h post-transfection, was two to three times as much as G1 and G1EF (Fig. 2B,D, middle panel).

Proteoglycan core proteins with newly attached GAG chains (neoproteoglycans) can be



% secreted after 12h	G1	G1EF	G1AA
	69	74	16
% in cell lysate after 12h	G1	G1EF	G1AA
	31	26	84

Fig. 2. Presence of transgenic proteins in spent media and cell lysates. Chinese hamster ovary (CHO) cells transfected with the transgenes in Figure 1B were continuously labeled with [^{35}S]methionine (**A** and **B**) or [^{35}S]sulfate (**C**), and incubated for the times indicated. A: Culture medium was collected, labeled transgenic proteins were isolated on nickel resin, eluted proteins were resolved on 5–15% gradient SDS/PAGE, and dried gels were exposed to film. G1 and G1EF transgenic proteins were present in spent medium at 4, 8, and 12 h. G1AA protein was only faintly visible as a lower molecular weight band at 12 h (lower arrowhead). B: Cell lysates were collected at the same time, and labeled transgenic proteins were resolved as above. Although all transgenic proteins were present in the lysate, there was significantly more G1AA present in the lysate at 12 h than in the medium at that time point. C: Neoproteoglycans were identified in the spent medium as described in ''Materials and Methods.'' Only G1 and G1EF were secreted as neoproteoglycans (brackets). **D**: Quantitative analyses of the autoradiographs in A, B, and C. The percent secreted was calculated as described in ''Materials and Methods.'' The results are from one selected representative experiment.

identified in culture medium by metabolically labeling cells with [^{35}S]sulfate. The sulfate is incorporated into the GAG chains as they are synthesized, thereby labeling the proteins they modify during processing in the Golgi. The transgenic proteins are further isolated from the spent medium with nickel resin chromatography that specifically recognizes the His6 tag. When separated on a gel, the GAG-modified, [^{35}S]sulfate containing neoproteoglycans appear as broad, smeared bands (Fig. 2C, bracket). Both the autoradiographs (Fig. 2C) and quantitative analyses (Fig. 2D, third panel) clearly show that G1 and G1EF were secreted as neoproteoglycans. There is no characteristically smeared band for G1AA. Therefore, G1 and G1EF secreted transgenic proteins were modified with the addition of complex oligosaccharide residues (presumably in the Golgi compartment) before secretion. Taken together, these results show that the majority of the AA-containing transgenic protein was localized inside the cell and not actively processed or secreted.

Intracellular Distribution of Transgenic Proteins

To establish the intracellular location of transgenic proteins, CHO cells were transfected with GFP-tagged transgenes for 14 h and then examined using epifluorescent microscopy. The distribution patterns of the different transgenic proteins in the cells were strikingly distinct (Fig. 3). Cellular G1-GAG-GFP as well as G1EF-GAG-GFP accumulated in conspicuous vesicles in the cytoplasm of transfected CHO cells (Fig. 3C,E). In contrast, G1AA-GAG-GFP was distributed diffusely throughout the cell with no evidence of vesicles (Fig. 3G).

To test if the transgenic protein distribution was due to the fact that CHO cells do

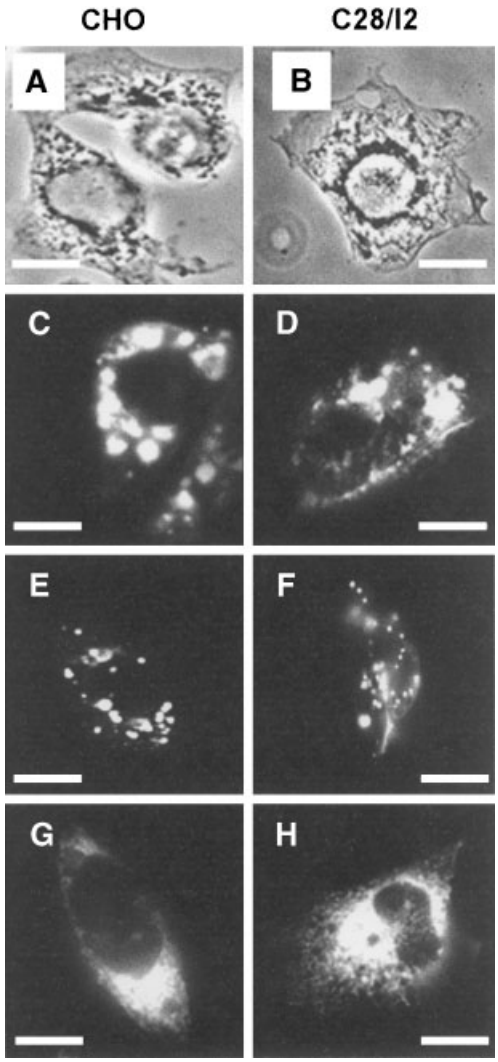


Fig. 3. Transgenic proteins distribute differently in the cell. CHO cells (**A**, **C**, **E**, **G**) and C28/I2 immortalized chondrocytes (**B**, **D**, **F**, **H**) were transfected with GFP-tagged transgenes. After 15 h, cells were fixed and images captured using epifluorescence. A, B: Phase contrast of cells shows normal phenotype. C, D: Cells transfected with G1-GAG-GFP have large vesicles containing GFP-expressing transgenic proteins. E, F: G1EF-GAG-GFP transfected cells also have large vesicles. G, H: G1AA-GAG-GFP, however, is diffusely distributed. The results are representative of four separate experiments. Bar, 10 μm.

not normally synthesize endogenous aggrecan, we repeated the same experiments in an immortalized human juvenile costal cartilage chondrocyte line (C-28/I2) that does synthesize aggrecan [Kokenyesi et al., 2000; Robbins et al., 2000]. The pattern of transgenic protein expression was similar to the pattern seen in CHO cells (Fig. 3D,F,H). Therefore, the intracellular distribution of the transgenic proteins is not due to unique features of CHO cells. The intracellular location of the transgenic protein is also not due to the addition of GFP to the transgenes because there was no difference in localization when fluorescently labeled His6 antibodies were used for detection (data not shown). The cellular localization patterns of G1-GAG-GFP and G1EF-GAG-GFP were always identical in CHO cells, so we will show only the G1EF-GAG-GFP results in the following studies.

Compartment Localization of Transgenic Proteins

In order to define the intracellular location of the transgenic proteins, cells transfected for 14 h were stained with markers for the ER and Golgi compartments (Fig. 4A–F). Many of the large G1EF-GAG-GFP containing vesicles had a "halo" of yellow suggesting that the transgenic proteins were located in the ER lumen with ER membrane-bound calnexin arround them (Fig. 4C and inset). G1AA-GAG-GFP was diffusely distributed with most of the diffuse pattern merging with the anti-calnexin antibody in the ER (Fig. 4F and inset). Live cells were imaged every hour after transfection with G1EF-GAG-GFP or G1AA-GAG-GFP so that we could observe the appearance of transgenic protein. At 3 h post-transfection, both transgenic proteins showed typical ER localization (Fig. 4G,I). Then at 4 h post-transfection, small but distinct vesicles containing transgenic protein appeared in G1EF-GAG-GFP transfected cells (Fig. 4H), but did not appear in G1AA-GAG-GFP transfected cells (Fig. 4J). Thus, the G1EF-GAG-GFP containing vesicles formed rapidly following transfection. When we examined transgenic protein localization in the Golgi compartment after 14 h (Fig. 5), the majority of G1EF-GAG-GFP transfected cells showed occasional regions of transgenic protein localized with mannosidase II, the Golgi marker (Fig. 5A). However, G1AA-GAG-GFP never localized in the Golgi (Fig. 5B).

Ultrastructural studies clarified the ER localization pattern of G1EF-GAG-GFP vesicles (Fig. 6). Accumulations of G1EF-GAG-GFP, labeled with anti-GFP antibodies and 10 nm gold particles, were seen in vesicle-like dilations in the lumen of the ER (Fig. 6A, arrows). G1AA-GAG-GFP transgenic protein also appeared in the ER, but the ER cisternae were normal (Fig. 6B, arrows). Therefore, the vesicular structures that we see in G1EF-GAG-GFP transfected cells are, in fact, enlarged portions of the ER.

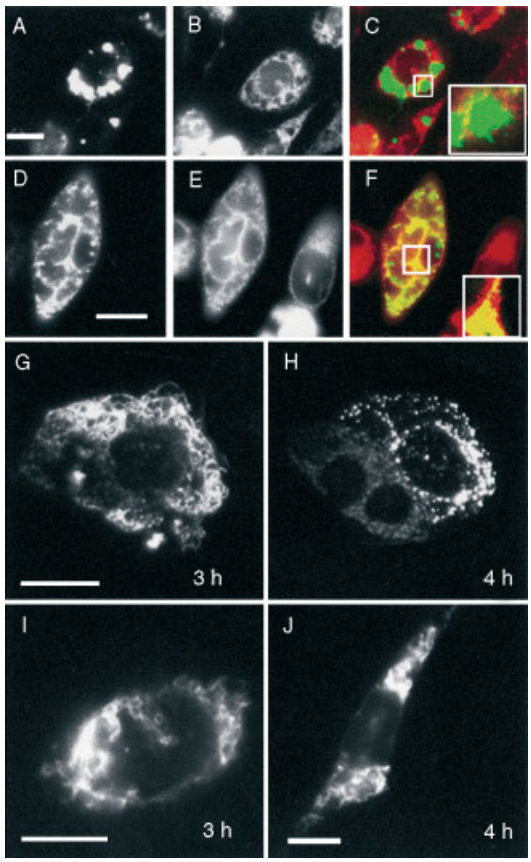


Fig. 4. ER localization of transgenic proteins in CHO cells. Cells were transfected with G1EF-GAG-GFP (**A**, **B**, **C**) or G1AA-GAG-GFP (**D**, **E**, **F**). After 15 h, cells were fixed, permeabilized, and incubated with anti-calnexin antibody (Cln), a marker for the ER, followed by Alexa 594-conjugated anti-rabbit antibody. The insets in (C) and (F) are magnified images of the outlined areas. The inset in (C) shows that there is a halo of yellow that surrounds the G1EF-GAG-GFP-containing vesicles denoting co-localization with the ER. The vesicles appear to actually displace portions of the ER. F: G1AA-GAG-GFP co-localizes extensively with the ER marker. Live CHO cells were imaged hourly after transfection; (**G**, **H**) vesicles began forming in G1EF-GAG-GFP transfected cells between 3 and 4 h post-transfection. These epifluorescent images are at different focal planes of the same cell. **I**, **J**: Live CHO cells transfected with G1AA-GAG-GFP showed a diffuse pattern in the ER by 3 h post-transfection and did not change after that. These are two separate cells. All images represent two separate experiments. Bar, 10 μm.

We tested whether the presence of GAG consensus sequences that are the sites of side chain modifications affected intracellular localization by transfecting CHO cells with representative transgenic constructs that did not contain GAG consensus sequences. The intracellular distributions of these transgenic proteins were identical to those seen when GAG sequences were present (data not shown). Thus, even though G1EF-GAG-GFP is modified with GAG chains in the Golgi (Fig. 2C), the presence or absence of modifications is not related to the vesicular accumulations in the ER.

In order to determine whether di-alanine could alter the localization pattern of G1EF-GAG-GFP, constructs G1EFAA-GAG-GFP and G1AAEF-GAG-GFP were made. It was interesting to see that G1EFAA-GAG-GFP localized with the same pattern as G1EF-GAG-GFP (compare Fig. 7A and Fig. 3E) and was secreted into spent medium (Fig. 7F, left panel) while G1AAEF-GAG-GFP localized with the same pattern as G1AA-GAG-GFP (compare Fig. 7B and Fig. 3G) and was not secreted (Fig. 7F, left panel). Apparently, di-alanine must be juxtaposed to the G1 domain in order to affect the ER localization pattern and secretion of the transgenic protein. In addition, when the G3 aggrecan core protein globular domain was included in the basic transgenes (G1-GAG-G3-GFP, G1EF-GAG-G3-GFP, and G1AA-GAG-G3-GFP), none of these transgenic proteins showed the vesicular accumulations (Fig. 7C–E, respectively). When the spent medium was analyzed for presence of secreted transgenic proteins, all of the proteins were present in spent medium (Fig. 7F, right panel). To summarize these data, the presence of AA next to G1 results in diffuse ER localization and impaired secretion. The absence of AA next to G1 results in an accumulation of transgenic proteins in large dilated regions of the ER, but those transgenic proteins are still secreted. The addition of the G3 globular domain appears to deter the accumulations of transgenic proteins in vesicles, and overcomes the secretory inhibition caused by the di-alanines juxtaposed to G1.

Since protein transport between the ER and the Golgi is mediated by microtubules [Presley et al., 1997], the requirement for intact microtubules in transgenic protein distribution was investigated. Tubulin polymerization was disrupted with nocodozole treatment during transgene transfection. After treatment, G1EF-GAG-GFP vesicles were still present (Fig. 8A) and G1AA-GAG-GFP localization patterns were not different from untreated cells (Fig. 8B), indicating that microtubules were not involved in localization. In addition, aggresome function relies on intact microtubules [Johnston et al., 1998] so that we can conclude that the vesicles are not aggresomes. Next, we disrupted the Golgi by incubating the transfected CHO cells

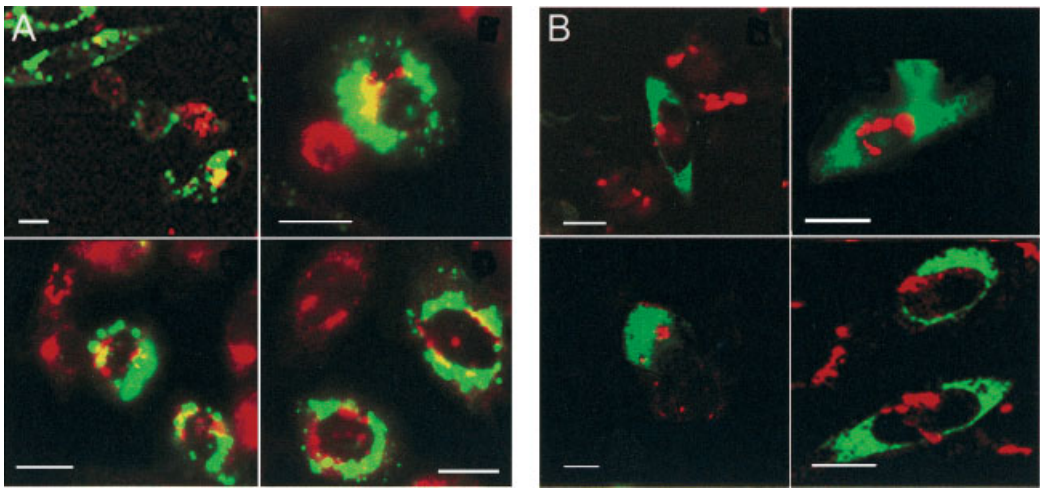


Fig. 5. Golgi localization of transgenic proteins in CHO cells. The cells were transfected with G1EF-GAG-GFP (**A**) or G1AA-GAG-GFP (**B**), and treated as described in Figure 4 using anti-mannosidase II antibody (MannII) as the marker for the Golgi. A: There is localization of G1EF-GAG-GFP in the Golgi as evidenced by the overlapping yellow portions of the merged images. B: G1AA-GAG-GFP did not localize in the Golgi at all. Confocal images are representative of four separate experiments. Bar, 10 μm.

with BFA. BFA will also induce recycling proteins to accumulate in the pre-Golgi compartment [Hauri et al., 2000]. Again, there was no effect on G1EF-GAG-GFP vesicle formation (Fig. 8C) and G1AA-GAG-GFP was still diffusely distributed in the ER (Fig. 8D). Likewise, incubating transfected cells at 15°C for 3 h, which halts anterograde traffic at the pre-Golgi compartment and stops retrograde traffic completely [Klumperman et al., 1998], had no effect on the intracellular distribution of the transgenic proteins (Fig. 8E,F). The same results were obtained when transfected cells were pretreated with cycloheximide to stop new protein synthesis before the 15°C incubation (data not shown). All together, these results indicate that G1EF-GAG-GFP vesicle formation occurs in the ER before transport to the Golgi, and G1AA-GAG-GFP localization is not due to recycling.

Transgenic Protein Association With β-CopI and CopII

Anterograde and retrograde transport between the ER and the Golgi is mediated by COPII- and COPI-coated vesicles, respectively [Barlowe, 2000]. We anticipated that soon-to-be-secreted G1EF-GAG-GFP (not in vesicles) would co-localize with COPII as it traveled from the ER to the Golgi, but not with COPI since there was no evidence of recycling. On the contrary, some of the G1EF-GAG-GFP vesicles did localize with COPI at 37°C (Fig. 9A,B) but not at 15°C (Fig. 9E,F) when trafficking stops and most cargo proteins including COPI and COPII accumulate in the Golgi and pre-Golgi compartments [Klumperman et al., 1998]. This would suggest that the G1EF-GAG-GFP vesicles do not exit the ER, and may even interact with COPI that transiently resides in the ER. And, in fact, the vesicles did not co-localize with COPII at 37°C (Fig. 9C,D) or 15°C (Fig. 9G,H), thus suggesting they never leave the ER. On the other hand, we postulated that if G1AA-GAG-GFP is exclusively localized in the ER, then there would be no co-localization of the transgenic protein with either COPI or COPII. As expected, at 37°C, there was no localization with either COPI (Fig. 9J,K) or COPII (Fig. 9L,M). Likewise, when the cells were incubated at 15°C, G1AA-GAG-GFP did not localize with COPI (Fig. 9N,O) or COPII (Fig. 9P,Q), indicating that G1AA-GAG-GFP did not exit the ER. Confocal microscopy confirmed the localizations (data not shown). Therefore, the results suggest that neither the vesicular accumulations of G1EF-GAG-GFP nor the ER lumenal G1AA-GAG-GFP transgenic proteins exited the ER by associating with COPII-coated vesicles. Vesicular accumulations of G1EF-GAG-GFP apparently remained in the ER and were not secreted as a distinct entity. Rather, secreted G1EF-GAG-GFP was extraneous to the vesicles and may have trafficked from ER to the Golgi compartment by a bulk flow mechanism.

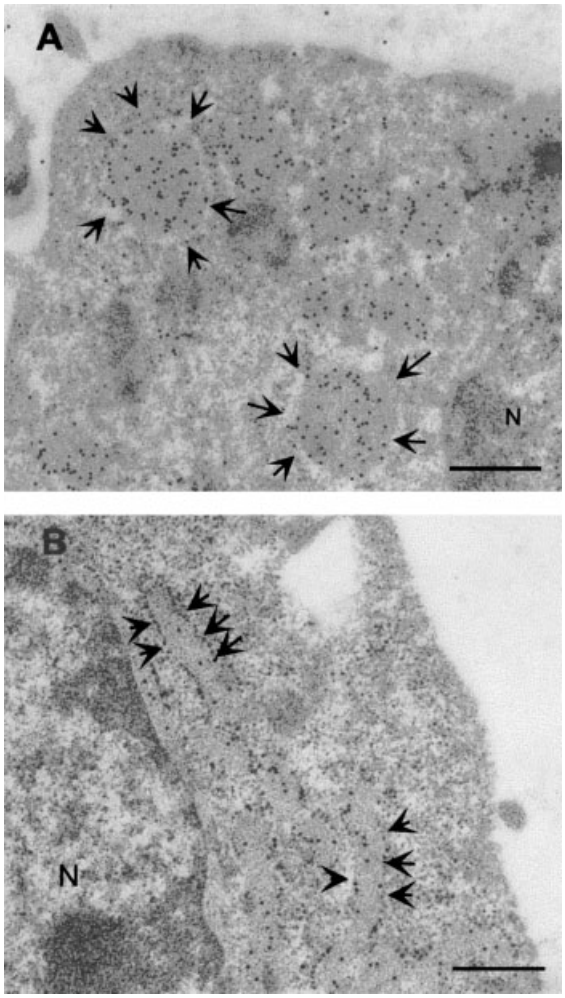


Fig. 6. Electron microscopic images of CHO cells transfected with either G1EF-GAG-GFP (**A**) or G1AA-GAG-GFP (**B**). Anti-GFP antibodies are tagged with 10 nm gold particles. Arrows in (A) surround distended ER cisternae containing transgenic protein. Arrows in (B) point to normal ER cisternae that contain transgenic protein. N, nucleus. Images are representative of two separate experiments. Bar, 0.5 μm.

DISCUSSION

This study in CHO cells shows that modifying the aggrecan core protein's G1 domain by juxtaposing di-alanine at its carboxyl terminus alters this domain's intracellular localization and secretion efficiency. G1AA-GAG-GFP transgenic proteins localized in the ER after translation (Fig. 3), were not modified in the Golgi and were not efficiently secreted (Fig. 2). Without the juxtaposed di-alanine, some portion of transgenic proteins, G1-GAG-GFP and G1EF-GAG-GFP, trafficked through the Golgi and was secreted into the culture medium (Fig. 2). However, there were also large, presumably unsecreted accumulations of these proteins in dilated regions of lumenal ER (Fig. 3). Although our previous studies showed that aggrecan's G3 domain is required for efficient secretion of aggrecan's core protein [Luo et al., 1996, 2001], the present study suggests that proteins containing only the G1 domain are, in fact, secreted at a slower rate partially due to their accumulation in the ER. The addition of the aggrecan core protein G3 domain to the transgenes reversed both the localization pattern of the G1EF transgenic proteins and, more importantly, the secretory behavior of the G1AA transgenic proteins (Fig. 7). These effects are further examples of the significance of the G3 domain to efficient secretion of aggrecan core protein.

The enlarged ER cisternae seen in G1-GAG-GFP and G1EF-GAG-GFP transfected cells are striking. Similar vesicles were reported as early as 1890, referred to as Russell Bodies (RBs), and described as dilated regions of the ER containing aggregations of misfolded, unsecreted proteins [Russell, 1890]. Later studies showed that RBs could be induced in secretory cells transfected with genes that coded for abnormal proteins and appeared as electron-dense ribosome-bounded bodies [Kopito and Sitia, 2000]. In contrast to RBs, though, the vesicles we see are not electron dense, but contain scattered transgenic protein (Fig. 6). The pattern is more accurately compared with the large distended portions of the ER seen in several diseases. For example, pseudoachondroplasia (PSACH) is characterized by abnormal ER retention of mutant cartilage oligomeric matrix proteins (COMP) in large inclusion bodies in chondrocytes [Hecht et al., 1998; Vranka et al., 2001]. The COMP mutations are either short sequence changes or deletions and result in localization of COMP and associated chaperones in enlarged ER cisternae [Hecht et al., 2001]. The skeletal defect, nanomelia, is caused by nonsecreted aggrecan, that is, retained in the ER due to a premature stop site in the GAG domain but, in contrast to G1EF-GAG-GFP, does not cause enlarged ER cisternae and is not secreted [Vertel et al., 1993]. Likewise, loss of function mutations of tyrosinase lead to its accumulation and retention in the ER of melanoma cells [Halaban et al., 1997, 2001]. The most common point mutation in the cystic fibrosis transmembrane conductance regulator protein (CFTR*F508) results in malfunction due to ER

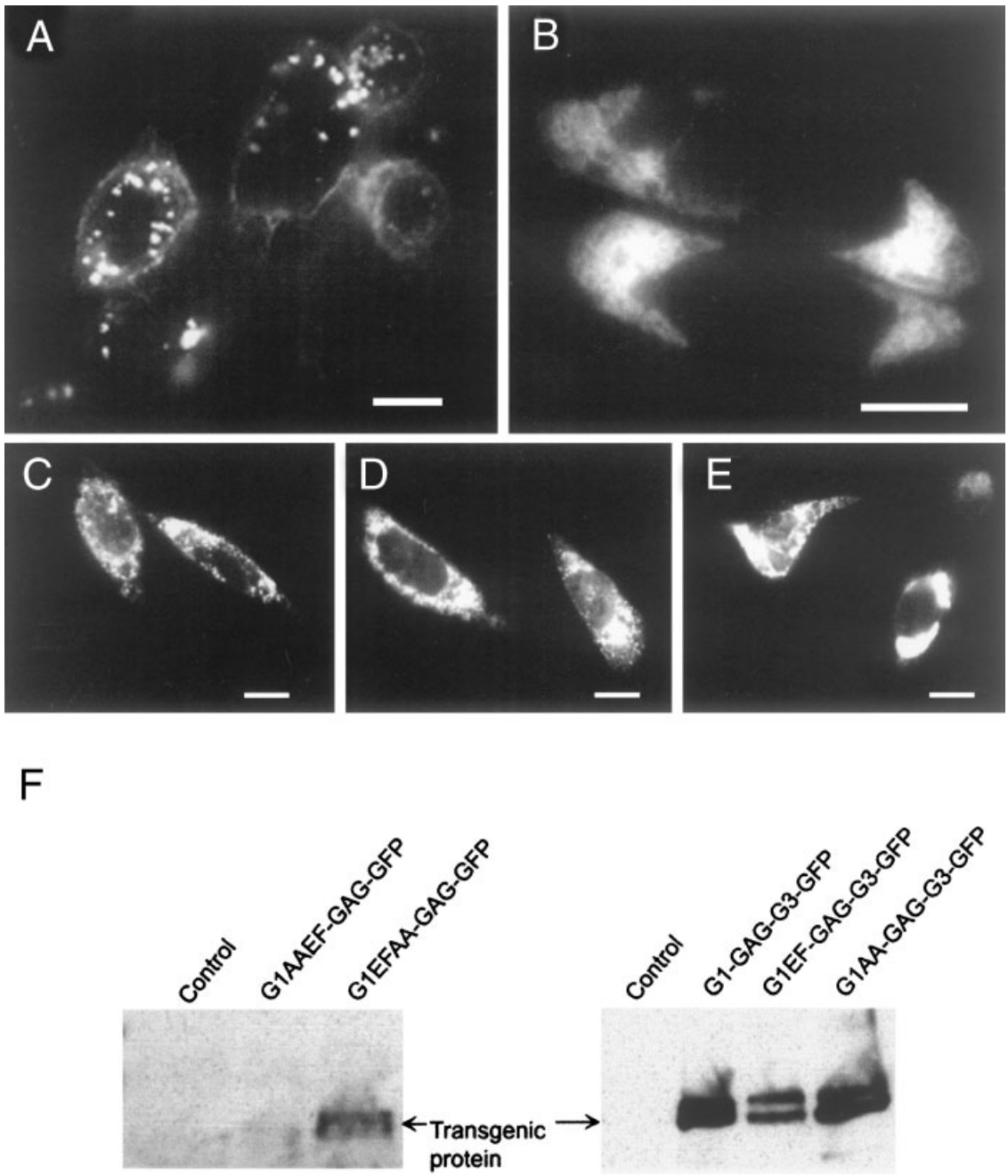


Fig. 7. Localization patterns of additional transgenic proteins. The confocal images of additional transgenic proteins show that G1EFAA-GAG-GFP (**A**) has the same vesicular intracellular distribution as G1EF-GAG-GFP, and G1AAEF-GAG-GFP (**B**) distributes throughout the ER like G1AA-GAG-GFP. When the aggrecan core G3 domain was added to produce G1-GAG-G3-GFP (**C**), G1EF-GAG-G3-GFP (**D**), and G1AA-GAG-G3-GFP (**E**), localization patterns of all the transgenic proteins were identical with no vesicles. **F**: Immunoblots (anti-His6 antibody) of concentrated spent medium from transfected cells. **Left panel** shows that transgenic protein G1EFAA-GAG-GFP was secreted but G1AAEF-GAG-GFP was not. **Right panel** shows that all of the G3-containing proteins were secreted. Blots are representative of two experiments. Confocal images are representative of three separate experiments. Bar, 10 μm.

retention [Haggie et al., 2002], and a single amino acid mutation of the protease inhibitor, α1-antitrypsin, causes accumulation and retention of that protein in the ER, resulting in α1-antitrypsin deficiency [Lomas et al., 1992; Teckman and Perlmutter, 2000; Primhak and Tanner, 2001]. As a matter of fact, the accumulations of mutant α1-antitrypsin in the ER of

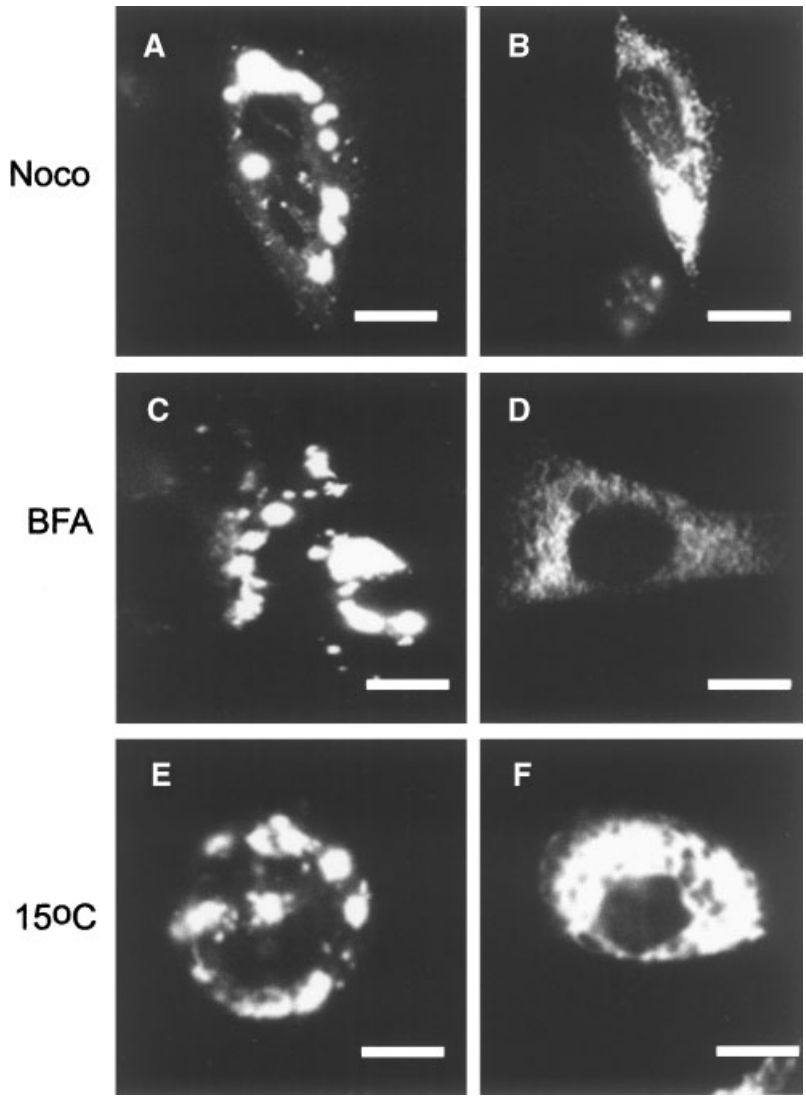


Fig. 8. Disruption of ER to Golgi transit does not affect the intracellular distribution patterns of transgenic proteins. CHO cells were transfected as before and either 10 μg/ml of nocodozole (noco) or 5 μg/ml of Brefeldin A (BFA) was added to the cultures before incubating for 14 h. After nocodozole treatment, G1EF-GAG-GFP (**A**) and G1AA-GAG-GFP (**B**) distributions were unchanged. **C**, **D**: Likewise, BFA treatment did not change intracellular distribution of the transgenic proteins. Cells were also incubated for 3 h at 15°C after 14 h transfection with the transgenes. The localization patterns of G1EF-GAG-GFP (**E**) and G1AA-GAG-GFP (**F**) were unchanged. Epifluorescent images represent three separate experiments. Bar, 10 μm.

hepatocytes [Carlson et al., 1989], as well as the COMP accumulations in PSACH chondrocytes [Hecht et al., 2001] look strikingly similar to the accumulations of G1-GAG-GFP and G1EF-GAG-GFP that we see in CHO cells.

There may be other explanations for why the expressed proteins without di-alanine accumulated in vesicles. Di-alanine juxtaposed to G1 might target the transgenic protein to the proteasome before it accumulates. In that case, the vesicles formed by the transgenic proteins without di-alanine would be similar to aggresomes. However, the nocodozole data preclude that possibility because aggresome formation is microtubule-dependent, and G1EF-GAG-GFP containing vesicles were present even after nocodozole treatment. Also, when we preincubated the cells with lactacystin to inhibit proteasome activity and aggresome formation, intracellular distributions of all transgenic proteins were unchanged (data not shown).

The localization and secretory behaviors of G1-GAG-GFP and G1EF-GAG-GFP transgenic proteins were of special interest. The majority of the transgenic proteins sequestered in ER vesicles was probably not secreted at all, since

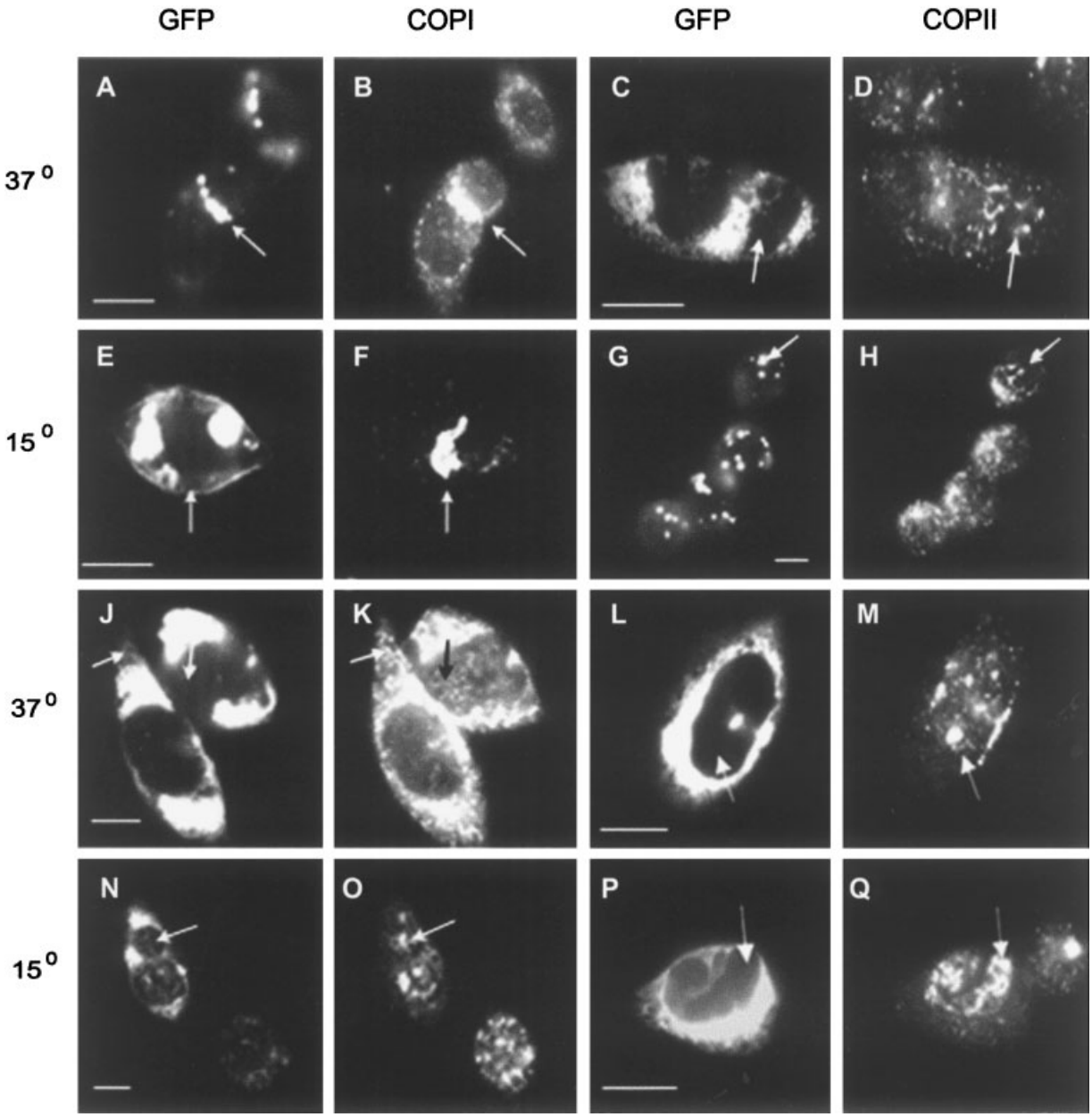


Fig. 9. Transgenic proteins and COP protein localization at 37 and 15°C. CHO cells were transfected for 14 h at 37°C with G1EF-GAG-GFP (**A–H**) and G1AA-GAG-GFP (**J–Q**) and then incubated at the indicated temperature for 3 h. After fixation, the cells were stained with antibody to either COPI (B, F, K, O) or COPII (D, H, M, Q). The only visible co-localization was G1EF-GAG-GFP with COPI at 37°C (A, B, arrows). Arrows in the remaining micrographs indicate unambiguous areas of non-co-localization. As expected, COPI and COPII redistributed to Golgi/pre-Golgi compartment areas following the 15°C incubation (F, H, O, Q). Epifluorescent images are representative of three experiments. Bar, 10 μm.

the vesicles were present even after microtubule disruption and BFA treatment. Therefore, the small amount of transgenic protein extraneous to the vesicles that trafficked through the Golgi compartment might be the only secreted protein. Since there was no evidence of co-localization with COPII (Fig. 9), a COPII-independent pathway, such as bulk flow, might mediate its transport to the Golgi.

In the case of G1AA-GAG-GFP, there are two possible explanations for the localization and secretory behavior. Either: (1) G1AA-GAG-GFP is retained in the ER by exclusion from COPII-coated vesicles [Scales et al., 1997]; or (2) it is

continuously recycled through the pre-Golgi and/or Golgi back to the ER in a retrograde COPI-coated transport vesicle [Barlowe, 2000]. The second explanation would indicate the presence of a gatekeeper in the pre-Golgi compartment that would return di-alanine-containing transgenic proteins to the ER before entering the Golgi. That is plausible because previous studies have identified a liver glycosyltransferase both in the ER and the pre-Golgi compartment that blocks transport of misfolded proteins to the Golgi [Zuber et al., 2001]. However, if G1AA-GAG-GFP cycled through the pre-Golgi, it would still be transported back to the ER in a COPI-coated vesicle which was not evident, nor did G1AA-GAG-GFP accumulate in the pre-Golgi at 15°C. Therefore, it seems more likely that the first explanation is valid, and the di-alanine juxtaposed to G1 creates a sequence that excludes it from COP-coated vesicles. Others have suggested that very small non-specific changes in protein sequences may result in activation of the ER quality control process, exclusion from COP-coated vesicles and retention of the chaperone-bound protein [Ellgaard and Helenius, 2003].

Because the di-alanine sequence only influences localization and inhibits secretion when it is directly adjacent to the globular G1 domain (Fig. 7A,B,F), the important sequence is most likely a combination of C-terminal G1 domain amino acids and di-alanine. The sequence FEALVPGAA (FEA), which includes the seven amino acids of the aggrecan G1 domain prior to the site of the di-alanine juxtaposition, is homologous to a sequence in the cytochrome P450 protein, CYP1B1. According to NCBI Sequence Viewer, the homologous region of the mouse protein is RESLVPGAA and of the human protein is CESLRPGAA, comprising amino acid residues 280 through 288. Interestingly, CYP1B1 is a nonsecreted transmembrane protein, that is, held in the ER membrane [Lodish et al., 2000]. It is possible that this sequence facilitates ER retention of G1AA-GAG-GFP as well, serving as a retention sequence. Another possibility is that the FEA sequence blocks binding with an accessory transport molecule like the GTP-binding protein Arf1 that mediates COPI binding [Presley et al., 2002] or with the Sec24 subunit of COPII [Lederkremer et al., 2001]. This is corroborated by the lack of G1AA-GAG-GFP localization with COPI and COPII vesicles, even when the cells are incubated at 15°C (Fig. 9). The fact that G1AA-GAG-GFP is not found in the Golgi indicates there is no COPII-independent transport as well. Thus, G1AA-GAG-GFP is similar to the C-terminal KKAA sequence, which is retained in the ER by a COPI-independent mechanism [Andersson et al., 1999].

One significance of this study is related to the importance of correctly assembled and secreted, full length aggrecan to the normal architecture of articular cartilage. By showing that aggrecan core protein mutations may cause abnormal accumulations in the ER and inefficient secretion, we can postulate that similar mutations might significantly hamper matrix protein secretion in vivo. A build-up of matrix proteins in the ER could lead to unfolded protein overload, induction of the ER stress response and eventually to the onset of apoptosis. In fact, our pilot studies in which immortalized chondrocytes were transfected with the constructs support this hypothesis because CHOP/GADD153 and ATF-4, both transducers of the ER stress response [Wang et al., 1996; Kawahara et al., 2001] were induced.

In conclusion, this study describes two important findings. First, juxtaposing a di-alanine C-terminal to the G1 domain of aggrecan transgenic proteins changes its secretory behavior in CHO cells. The presence of di-alanine in the transgenic protein minimizes its secretion and results in its localization in the ER whereas the absence of di-alanine results in a transgenic protein which is secreted. Second, excess transgenic protein without di-alanine accumulates in vesicles localized in the ER while the presence of the di-alanine adjacent to the globular domain inhibits formation of the vesicles. These results indicate that juxtaposed di-alanine profoundly affects a large globular protein's intracellular localization.

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