Degradation of aggrecan precursors within a specialized subcompartment of the chicken chondrocyte endoplasmic reticulum

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Chicken chondrocytes in culture synthesize aggrecan proteoglycan as a 370 kDa precursor that is glycosylated and secreted into the medium with a half-life of 30 min. In metabolic studies the 370 kDa precursor was shown to render a degradation intermediate of 190 kDa, which appeared with no measurable lag phase; it was dependent on temperature (> 20 °C) and inhibited by certain serine and serine/cysteine protease inhibitors such as leupeptin and PMSF. By contrast, degradation was unaffected by treatment of the cells with brefeldin A or with lysosomotropic agents. Aggrecan precursors were detected by immunofluorescence analysis within a subcompartment of the endoplasmic reticulum (ER), previously characterized as a smooth-membrane-bound subregion [Vertel, Velasco, LaFrance, Walters and Kaczman-Daniel (1989) J. Cell Biol. **109**, 1827–1836]. Analysis of the subcellular fractions derived from chondrocytes indicated that the degradation intermediate was concentrated in the ER subcompartment. Degradation was dependent on the Ca^{2+} concentration and the redox state in the ER. Treatment of the cells with agents or conditions that alter the degradation rate of aggrecan precursors, such as protease inhibitors, decreased temperature or dithiothreitol, also modified the retention of these molecules in the ER subcompartment, as seen by immunofluorescence. These results indicate that a fraction of the 370 kDa aggrecan precursor is targeted to a smooth ER subcompartment where it undergoes degradation.

INTRODUCTION

Proteins destined to enter the exocytic pathway are synthesized in the endoplasmic reticulum (ER). Rough (ribosome-studded) and smooth cisternae account for most of the ER which is increasingly recognized to include a number of functionally distinct subcompartments [1]. Distinct ER subcompartments involved in protein glycosylation [2,3], virus assembly [4], retention of misfolded or aggregated proteins [5–8] and Ca^{2+} regulation [9–11] have been described.

The ER is also the site where misfolded polypeptides or unassembled subunits of multimeric proteins are degraded [12]. In general, proteins that fail to fulfil the requirements for transport out of the ER can be degraded in this organelle. This provides a mechanism for the early disposal of molecules not meeting functional and/or conformational requirements. It is also possible that cellular production of certain proteins is regulated through control of synthesis and degradation rates in the ER. However, it is unknown how these activities are coordinated. One possibility is that proteolysis occurs in particular subcompartments of the ER [13]. In a previous study we reported that precursors of chondroitin sulphate (aggrecan) proteoglycans accumulate in a specialized ER subcompartment of cultured chondrocytes [14]. The present study demonstrates that degradation of aggrecan precursors occurs in this subcompartment and provides evidence for the compartmentalization of the proteolytic activities within the ER.

MATERIALS AND METHODS

Antibodies and chemicals

Aggrecan monomers were purified from chick embryo sterna by a previously published method [15] and used to immunize a rabbit. The antiserum was precipitated with 50 % $(NH_4)_2SO_4$ and purified on an aggrecan–Sepharose 4B column. It recognized aggrecan core protein in immunoblots and immunoprecipitates of total cellular proteins. The antibody was further purified on proteoglycan bands transferred to Immobilon P membrane (Millipore, Bedford, MA, U.S.A.) as described by Olmsted [16] using 0.2 M glycine, pH 2.8, to elute bound immunoglobulins and 1 M Tris, pH 8.0, to neutralize eluents. A mouse monoclonal antibody against keratan sulphate was purchased from ICN Biochemicals (High Wycombe, Bucks., UK). Monoclonal antibody against the KDEL sequence was a gift from Dr. S. Fuller (European Molecular Biology Laboratory, Heidelberg, Germany). Endoglycosidases H and D (endo H and D) were purchased from Boehringer, Mannheim, Germany and chondroitinase ABC from Seikagaku Kogyo Co. Brefeldin A was from Epicenter Technologies (Madison, WI, U.S.A.). Protease inhibitors were obtained from either Sigma (St. Louis, MO, U.S.A.) or Boehringer. Chloroquine, monensin, A23187, cycloheximide, diamide, dithiothreitol (DTT), saponin and BSA were purchased from Sigma.

Cell culture

Tissue culture products were purchased from Gibco/BRL (Gaithersburg, MD, U.S.A.). Chondrocytes were isolated from the sterna of 14-day-old chicken embryos as previously described [3]. Cells were maintained in Ham's F-12 medium supplemented with 10 % (v/v) foetal calf serum, 2 mM glutamine, 50 μg/ml streptomycin and 50 units/ml penicillin. For metabolic labelling, cells were maintained in suspension in 15 cm Petri dishes. Cells for immunofluorescence were attached to gelatinized carbon-coated coverslips. In both cases, cultures were fed with fresh

Abbreviations used: ER, endoplasmic reticulum; endo H, endo-β-*N*-acetylglucosaminidase H; endo D, endo-β-*N*-acetylglucosaminidase D; DTT, dithiothreitol; ALLM, *N*-acetyl-leucyl-leucyl-methioninal; ALLN, *N*-acetyl-leucyl-leucyl-norleucinal.

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medium every other day and 1 h before start of the experiments on day 4.

Metabolic labelling and immunoprecipitation

Cells were recovered by centrifugation (1000 ***g*** for 10 min) and treated with trypsin (5 min; 37 °C) to disrupt cellular aggregates. For pulse–chase experiments, 0.5×10^6 cells/ml were incubated in methionine- and cysteine-free MEM medium (Sigma) containing 10 % (v/v) dialysed foetal calf serum for 20 min, pulsed for 5 min in the same medium containing 200 μCi/ml Tran^{35}S-label (1000–1100 Ci/mmol; ICN Radiochemicals, Cleveland, OH, U.S.A.), and chased in complete medium containing a 100-fold excess of both unlabelled methionine and cysteine. Cells and media were separated by centrifugation (1000 ***g***; 10 min) and the cell pellets were rinsed twice in cold PBS. Cells were then lysed in 50 mM Tris/HCl buffer, pH 8.0, containing 400 mM NaCl, 5 mM EDTA, 1 % (v/v) Triton X-100 and protease inhibitors (5 mM benzamidine, 1 mM PMSF, 100 μg/ml soybean trypsin inhibitor). Both media and postnuclear supernatants derived from cell lysates were first cleared by incubation with preimmune rabbit serum (1:40 dilution) for 1 h at room temperature followed by 30 min incubation with preswollen Protein A–Sepharose (10 mg/sample; Sigma). Samples were centrifuged at 3000 ***g*** for 30 s and the supernatants transferred to new Microfuge tubes. Antiserum against aggrecan was added (1:100 dilution) and the incubation continued for 1 h at room temperature or overnight at 4 °C. Samples were then incubated with Protein A–Sepharose, as above. Immunoprecipitates from cell lysates were sequentially rinsed three times in regular lysis buffer lacking protease inhibitors, twice in lysis buffer containing 800 mM NaCl, and twice in 10 mM Tris/HCl buffer, pH 7.5. They were then denatured and subjected to SDS/PAGE (5 % acrylamide gels) and fluorography as described [3]. Band intensities on autoradiographs were determined by scanning densitometry using Molecular Dynamics model 300A densitometer (Sunnyvale, CA, U.S.A.); data were analysed using the Image Quant Software (V 3.0, 1991; Molecular Dynamics). In studies involving calculation of aggrecan intracellular retention the 370 kDa precursor band localized on autoradiograms from pulse-labelled cells was excised from dried gels, rehydrated, dissolved in H_2O_2/NH_4OH [17], and quantified by scintillation counting. Immunoprecipitates from media were washed as above, rinsed in 90 % (v/v) acetone/10 % (v/v) HCl, dried overnight at 60 °C, and their radioactivity quantified by scintillation counting. Endoglycosidase treatment of immunoprecipitates was performed at 37 °C for 16 h as described [18].

Subcellular fractionation

All steps were performed at 4 °C. Radiolabelled chondrocytes (10^8–10^9 cells) were resuspended in a small volume (3–4 ml) of 10 mM Tris/HCl, pH 7.4, containing 10 mM KCl, 2.5 mM $MgCl_2$, 250 mM sucrose and 5 mM benzamidine. The cell suspension was passed 9–12 times through a ball-bearing homogenizer (H & Y Enterprise, Redwood City, CA, U.S.A.) and then centrifuged at 600 ***g*** for 10 min. The nuclear pellet was rinsed and centrifuged again. Postnuclear supernatants were pooled and centrifuged at 12000 ***g*** for 15 min. The postmitochondrial supernatant was either incubated or not with 25 mM EDTA for 1 h before centrifugation at 100000 ***g*** for 1 h (SW60 rotor; Beckman Instruments, Palo Alto, CA, U.S.A.). Microsomes were resuspended in 0.5 ml of 52 % sucrose. This solution was placed on top of a 61 % sucrose cushion (0.5 ml) at the bottom of an SW60 ultracentrifuge tube. It was overlaid with 46 % (0.5 ml), 38 % (0.5 ml), 30 % (0.75 ml), 26 % (0.75 ml) and 20 % (0.5 ml) sucrose solutions prepared in distilled water. Centrifugation was performed at 120000 ***g*** for 12 h. Fractions (400 μl) were collected from the bottom of the tube and used to determine both refraction index and RNA content. In addition, *p*-nitrophenyl-α-D-mannosidase activity at pH 5.5 was determined as described by Tulsiani et al. [19]. Membranes in each fraction were pelleted at 120000 ***g*** for 1 h in a TLA-100 ultracentrifuge (Beckman Instruments). They were resuspended in 0.5 ml of 50 mM Tris/HCl buffer, pH 8.0, containing 400 mM NaCl, 5 mM EDTA, 1 % (v/v) Triton X-100 and 1 mM PMSF and processed for aggrecan immunoprecipitation, SDS/PAGE and fluorography.

Immunofluorescence microscopy

Monolayers of chondrocytes were rinsed twice in PBS. The extracellular matrix was digested with 1 mg/ml hyaluronidase for 10 min at 37 °C. Cells were then fixed in 3 % formaldehyde in 0.1 M phosphate buffer, pH 7.2, for 30 min at room temperature. Alternatively, cells were treated in a CO_2 incubator for 2 h at 37 °C with culture medium containing either DTT, cycloheximide or protease inhibitors before fixation. In other experiments, the cells were incubated at low (15 or 20 °C) temperature in medium supplemented with 20 mM Hepes or at 37 °C for 3 h with 600 μg/ml rhodamine/ovalbumin (Molecular Probes Inc., Eugene, OR, U.S.A.) in serum-free medium. After fixation the cells were rinsed in PBS, permeabilized with 0.05 % (w/v) saponin in PBS, and incubated with primary antibodies. Antibodies were diluted in PBS/0.5 % (w/v) BSA/0.05 % (w/v) saponin. Incubation with antibodies was performed at room temperature for 1 h. Primary antibodies were detected in a second incubation step with either fluorescein-labelled goat anti-rabbit IgG or rhodamine-labelled goat anti-mouse IgG (TAGO, Burlingame, CA, U.S.A.). Unbound antibodies were washed out with PBS and the coverslips were mounted in PBS/glycerol.

RESULTS

Identification of intracellular proteins related to aggrecan core protein

Cultured chondrocytes were radiolabelled for 5 min with Tran^{35}S-label, lysed and subjected to immunoprecipitation with an antibody against aggrecan monomer. The antibody recognized a 370 kDa band previously described as the major aggrecan core protein precursor present within cells (Figure 1A, lane 3) [20]. In cells chased for 60 min with unlabelled methionine and cysteine an additional band of 190 kDa was also recognized specifically by the antibody (Figure 1A, lane 4). This protein was shown to be immunologically related to the aggrecan molecule since it was immunoprecipitated by affinity-purified antibody on the 370 kDa band (Figure 1A, lane 5). Conversely, affinity-purified antibody on the 190 kDa band also immunoprecipitated the 370 kDa precursor (not shown). In cells chased for short times (10–15 min) the crude polyclonal antibody also bound an additional 180 kDa band which was not studied further. In contrast, other minor bands were shown to be non-specific as they also appeared in control samples incubated with preimmune serum (Figure 1A, lane 2) or non-incubated with antibody (Figure 1A, lane 1). Only aggrecan proteoglycans were immunoprecipitated from the medium of pulse-labelled cultures chased for 60 min (Figure 1B, lane 1). Their high molecular mass prevented them from entering the stacking gel, which could be achieved after chondroitinase treatment (Figure 1B, lane 2).

Intracellular degradation of aggrecan precursors

The processing of aggrecan core protein precursors was analysed by pulse–chase experiments. Cells were metabolically labelled

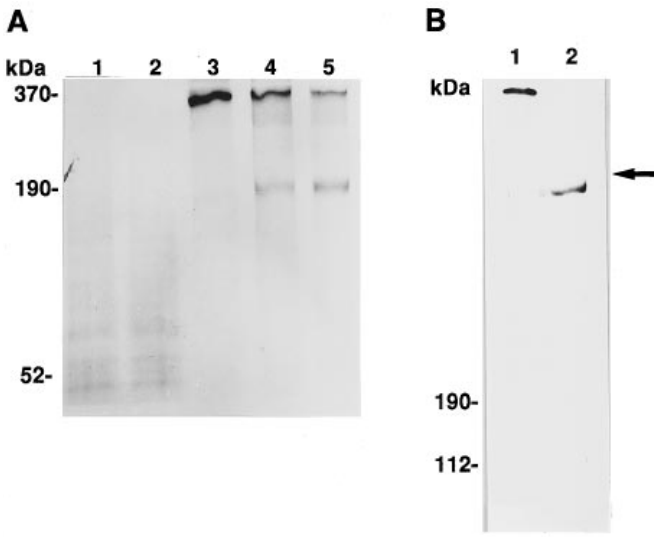

Figure 1 Specificity of the antibody against aggrecan proteoglycan

Chondrocytes were pulse-labelled with 200 μCi/ml Tran^{35}S-label for 5 min and chased at 37 °C for 60 min in the presence of unlabelled methionine and cysteine. (**A**) Lysates from both pulse-labelled (lanes 1–3) and chased cells (lanes 4–5) were subjected to immunoprecipitation as described in the Materials and methods section using no primary antibody (lane 1), preimmune antiserum (lane 2), rabbit anti-aggrecan serum (lanes 3 and 4) or anti-aggrecan antibody preabsorbed on Western blots containing the 370 kDa precursor (lane 5). (**B**) Immunoprecipitates from the tissue culture medium of chased cells were incubated (lane 2) or not (lane 1) with chondroitinase ABC before SDS/PAGE. The arrow indicates the limit between the stacking and resolving gels.

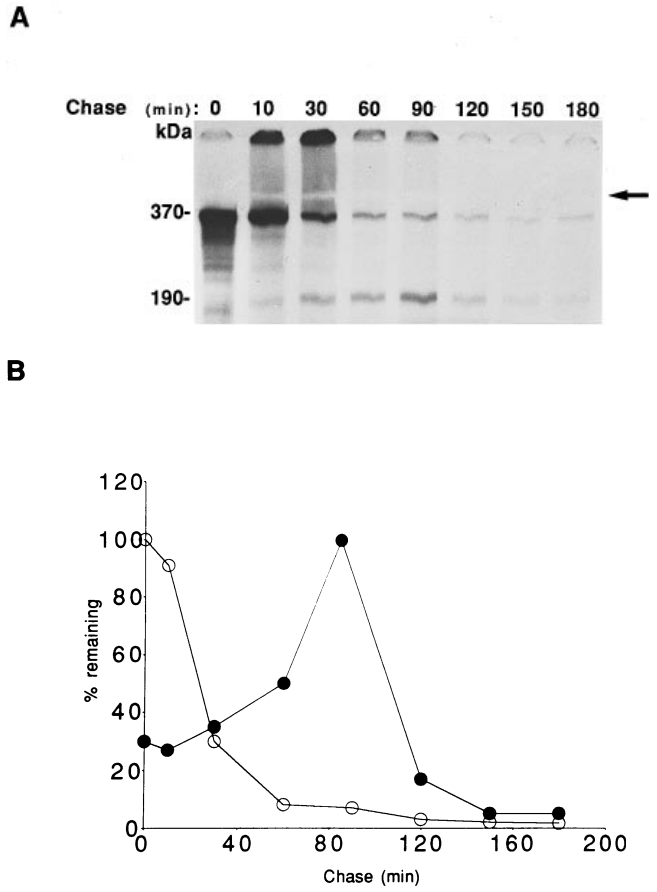

Figure 2 Pulse–chase analysis of *de novo* synthesized aggrecan core protein precursors in cultured chondrocytes

Cells were pulse-labelled with 200 μCi/ml Tran^{35}S-label for 5 min and chased in unlabelled medium at 37 °C for the indicated time periods. Aggrecan precursors were immunoprecipitated from cell lysates, resolved by SDS/PAGE, and detected by fluorography. Bands corresponding to the 370 kDa precursor (○) and the 190 kDa intermediate (●) were located on autoradiogram (**A**) and quantified by scanning densitometry (**B**). The limit between the stacking and resolving gels is indicated (arrow). Values are plotted as a percentage of the maximal amount of each molecular form found within cells. Data are representative of three different pulse–chase experiments.

with Tran^{35}S-label for 5 min and chased for different periods. Aggrecan-related proteins were immunoprecipitated from both cell lysates and culture medium using the anti-aggrecan antibody. Newly synthesized aggrecan core protein precursor was detected as a 370 kDa precursor that decreased with a half-life of approx. 25–29 min (Figure 2). Higher-molecular-mass forms appearing at the top of the stacking gel probably represent heavily glycosylated aggrecan monomers that were in the process of being secreted from the Golgi complex. Accordingly, aggrecan monomers were immunoprecipitated from the culture medium with a half-life of 30 min (Figure 3). In addition, the 190 kDa intracellular form increased transiently during the first 90 min of the chase and then decreased gradually (half-life of 108 min) (Figure 2A). This suggested the possibility that the 190 kDa band represents a degradation intermediate derived from the 370 kDa precursor. In fact, only 73–75 % of the total radioactivity initially incorporated by the cells into the 370 kDa precursor during a 5 min pulse was recovered from the culture medium as ^{35}S-aggrecan monomers after a 3 h chase period. Therefore we inferred that 25–27 % of the pulse-labelled aggrecan precursors were retained intracellularly and that degradation was probably the fate of these molecules since more than 98 % of them disappeared from within cells during this chase period. As judged from the appearance of the 190 kDa intermediate, the initial degradation step occurred with no apparent lag.

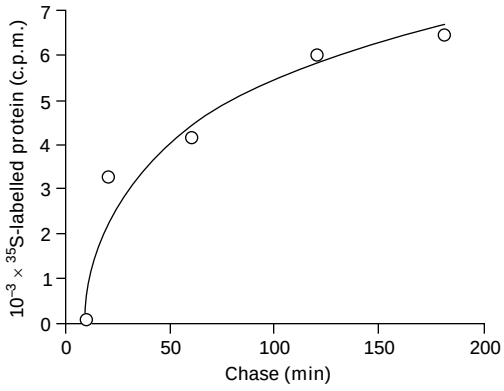

Figure 3 Secretion of mature aggrecan molecules by cultured chondrocytes

Cells were pulse–chased as indicated in Figure 2. Secreted aggrecan monomers were immunoprecipitated from the medium at various time points and quantified by scintillation counting.

Serine/cysteine proteases degrade aggrecan precursors in a pre-Golgi compartment

In order to determine whether the 190 kDa form was indeed a degradation intermediate, we incubated pulse-labelled chondrocytes with different protease inhibitors at concentrations that

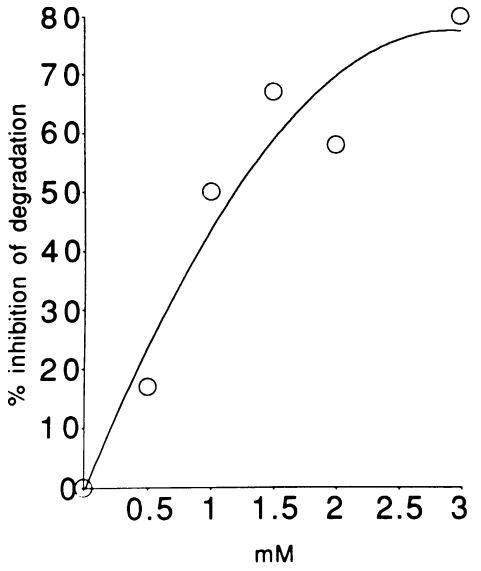

Figure 4 Inhibition of aggrecan core protein intracellular degradation by PMSF

Pulse-labelled chondrocytes were chased for 90 min at 37 °C in the presence of the indicated concentrations of PMSF. Cell lysates and medium samples were immunoprecipitated with anti-aggrecan antibody and processed as indicated in Table 1. Values are plotted as percentage degradation inhibition compared with control cells incubated in chase medium with no inhibitor. A similar curve was obtained for cells treated with leupeptin but in this case the concentrations needed to achieve significant inhibition were two to three times lower.

do not affect cell viability. After a 90 min chase, aggrecan-related molecules were immunoprecipitated from both cell lysates and medium samples. Intracellular accumulation of the 370 kDa aggrecan core protein precursor was taken as an indication of degradation inhibition for those treatments that do not interfere with secretion of aggrecan monomers in comparison with control untreated cells. Thus degradation was substantially inhibited in cells chased in the presence of certain serine/cysteine protease inhibitors such as PMSF and leupeptin (Figure 4). Neither PMSF nor leupeptin, however, inhibited secretion of mature aggrecan monomers into the culture medium (Table 1). Degradation inhibition was also observed with Tos-Lys-CH_2Cl and Tos-Phe-CH_2Cl but these serine protease inhibitors also affected processing and/or secretion of aggrecan monomers. In contrast, degradation was unaffected by inhibitors of metalloproteases (EDTA, phosphoramidon), cysteine proteases (ALLM and ALLN), aminopeptidases (bestatin), acid proteases (chymostatin and pepstatin A) or cathepsin A and B (antipain) (Table 1).

The cellular location of the 190 kDa intermediate was investigated by testing the sensitivity of immunoprecipitated proteins to treatment with endoglycosidases. Both the 190 kDa intermediate and the 370 kDa aggrecan precursor were sensitive to endo H treatment (Figure 5A, lane 3), but resistant to endo D treatment (Figure 5A, lane 2). Since this is characteristic of glycoprotein precursors that have not reached the Golgi complex [21,22], these results indicated that degradation of the 370 kDa aggrecan precursor giving rise to the 190 kDa intermediate occurred in a pre-Golgi location. Further support for this conclusion was obtained from experiments with cells chased in the presence of brefeldin A. As shown in Figure 5(B), degradation still occurred in the presence of brefeldin A indicating that it was not affected by the disruption of the Golgi complex caused by this drug [23]. In addition, we examined whether degradation of aggrecan

Table 1 Effect of different protease inhibitors on the secretion and ER degradation of aggrecan precursors

Cells (5×10^6 for each treatment) were pulse-labelled with 200 μCi/ml Tran^{35}S-label for 5 min and chased for 90 min at 37 °C in the presence of one of the protease inhibitors indicated below. Cell lysates and medium samples were subjected to immunoprecipitation with anti-aggrecan antibody. Immunoprecipitates from cells were resolved by SDS/PAGE (same number of counts per lane were loaded), detected by fluorography, and the 190 kDa and 370 kDa bands analysed by scanning densitometry. Immunoprecipitates from tissue culture medium as well as the 370 kDa band from the pulse sample were quantified by scintillation counting. Protein retention was calculated by subtracting the amount of aggrecan secreted into the medium during the chase period from the amount of 370 kDa precursor synthesized during the pulse. The percentage of aggrecan retained intracellularly is shown. Inhibition of aggrecan degradation was calculated as a decrease in the percentage of the p190 form with respect to the 370 kDa precursor, compared with control cells incubated in chase medium with no inhibitor. Values for the 370 kDa precursor were corrected for the intracellular accumulation of aggrecan precursors observed in each treatment. ALLM, *N*-acetyl-leucyl-leucyl-methioninal; ALLN, *N*-acetyl-leucyl-leucyl-norleucinal. Data are representative of at least three different determinations.

Treatment	Protein retention (%)	Inhibition of degradation (%)
Control	27.0	0
EDTA (5 mM)	42.3	11.3
Phosphoramidon (200 μg/ml)	34.7	14.3
Aprotinin (20 μg/ml)	33.0	0
PMSF (1 mM)	30.0	45
Leupeptin (200 μg/ml)	33.3	53.1
Tos-Lys-CH_2Cl (100 μM)	62.2	62.8
Tos-Phe-CH_2Cl (20 μM)	64.8	35.3
Antipain (200 μg/ml)	34.2	15.5
ALLM (200 μg/ml)	49.0	13.5
ALLN (200 μg/ml)	22.0	3
Chymostatin (200 μg/ml)	37.4	11.7
Pepstatin A (200 μg/ml)	38.8	10.4
Bestatin (200 μg/ml)	27.2	0

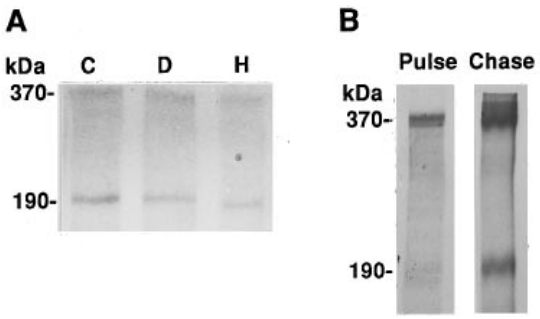

Figure 5 Demonstration of intracellular degradation of aggrecan core protein precursors in a pre-Golgi compartment

(**A**) Aggrecan-related proteins were immunoprecipitated from cell lysates of chondrocytes labelled for 30 min with 200 μCi/ml Tran^{35}S-label and subjected to digestion with endoglycosidases (H or D) or mock digested (C). Immunocomplexes were fractionated by SDS/PAGE and detected by fluorography. (**B**) Autoradiogram showing aggrecan-related molecules immunoprecipitated from pulse-labelled cells chased for 3 h at 37 °C in the presence of 2 μg/ml brefeldin A.

precursors was affected by pretreatment of the cells with agents that interfere with endosome and lysosome function. Although some accumulation of the 370 kDa precursor was observed in cells preincubated with NH_4Cl or chloroquine, this was probably the result of a decreased rate of transport through the Golgi (Table 2) rather than inhibition of degradation. Thus no signi-

Table 2 Effect of various treatments on the ER degradation of aggrecan precursors

Cells (5×10^6 for each treatment) were metabolically labelled with 200 μCi/ml Tran^{35}S-label for 5 min and then incubated at 37 °C for 3 h in chase medium supplemented with the compounds indicated below or incubated in chase medium at low temperature (15 or 20 °C). Secreted aggrecan molecules were immunoprecipitated from the medium and quantified by scintillation counting. Intracellular aggrecan-related molecules were also immunoprecipitated, resolved by SDS/PAGE, detected by fluorography, and quantified by densitometric scanning of autoradiograms. Data are means of three different experiments.

	Protein retention (%)	Inhibition of degradation (%)
Control	26	0
Inhibition of ER-to-Golgi transport		
Brefeldin A (2 μg/ml)	94.6	4
Monensin (100 μM)	56	27
Inhibition of lysosomal degradation		
NH_4Cl (50 μM)	71.5	13.4
Chloroquine (100 μM)	43	7
Energy-depletion treatments		
20 mM NaN_3 + 5 mM NaF	3.5	0
40 mM NaN_3 + 10 mM NaF	30	0
Low-temperature incubations		
15 °C	70	35
20 °C	44	37
Inhibition of protein synthesis		
Cycloheximide (100 μg/ml)	23	21

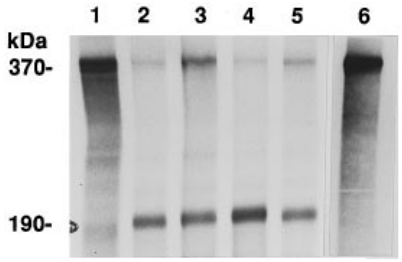

Figure 6 Demonstration that lysosomotropic agents do not inhibit intracellular degradation of aggrecan core protein precursors

Pulse-labelled cells (lane 1) were chased for 90 min at 37 °C in unlabelled medium containing 50 mM NH_4Cl (lane 3), 100 μM chloroquine (lane 4) or 100 μM monensin (lane 5), agents known to raise the intralumenal pH of lysosomes and endosomes and thus inhibit protein degradation in these organelles. For comparison, cells similarly chased in control medium with no inhibitor (lane 2) or in medium containing 200 μg/ml leupeptin (lane 6) were also processed. Aggrecan-related proteins were immunoprecipitated from cells and subjected to SDS/PAGE and fluorography.

ficant decrease in the 190 kDa intermediate was observed during these treatments (Figure 6, lanes 2–5).

Degradation intermediates are concentrated in a specialized ER subcompartment

It has been shown previously that aggrecan core protein precursors accumulate in a distinct smooth subregion of the ER continuous with typical rough cisternae [14]. Therefore we examined the possibility that degradation of aggrecan precursors occurred in that subcompartment. The subcompartment was identified by immunofluorescence analysis as peripheral vesicles that did not stain with an antibody against keratan sulphate

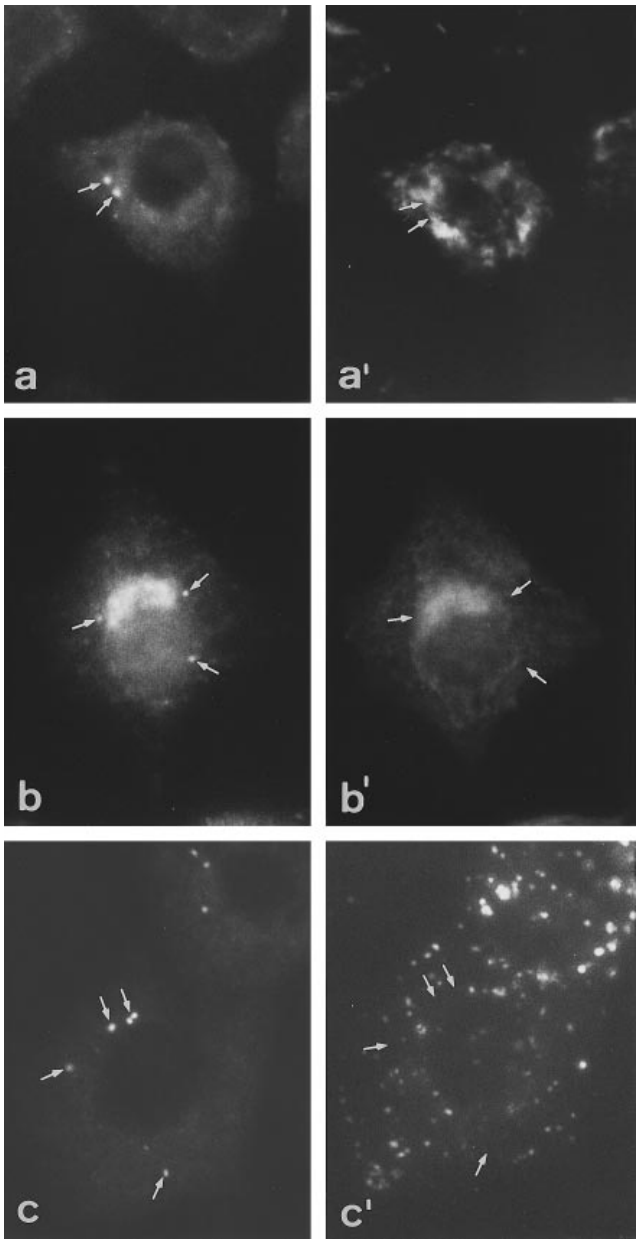

Figure 7 Localization of aggrecan-related proteins in chondrocytes

Monolayers of chondrocytes were fixed and permeabilized (see the Materials and methods section). Cells were processed for immunofluorescence by simultaneous staining with antibodies against aggrecan (**a**, **b**), and either KDEL (**a**′) or keratan sulphate (**b**′). Alternatively, before fixation, cells were incubated at 37 °C for 3 h in serum-free medium containing 600 μg/ml rhodamine/ovalbumin. They were then permeabilized and stained with anti-aggrecan (**c**) to compare the intracellular distribution of aggrecan-related molecules with that of endocytosed rhodamine/ovalbumin (**c**′). Arrows in each double-picture set indicate the locations of ER subcompartments containing aggrecan precursors. Magnifications: × 400.

(Figures 7b and 7b′), a glycosaminoglycan modification acquired by aggrecan core protein precursors during their transport through the Golgi complex [24]. The vesicles, however, did stain with an antibody against the C-terminal sequence KDEL (Figures 7a and 7a′), the retention signal for many ER lumenal proteins [25]. The ER subcompartments containing aggrecan-related molecules were readily distinguished from endosomes and lysosomes preloaded with endocytosed rhodamine/ovalbumin

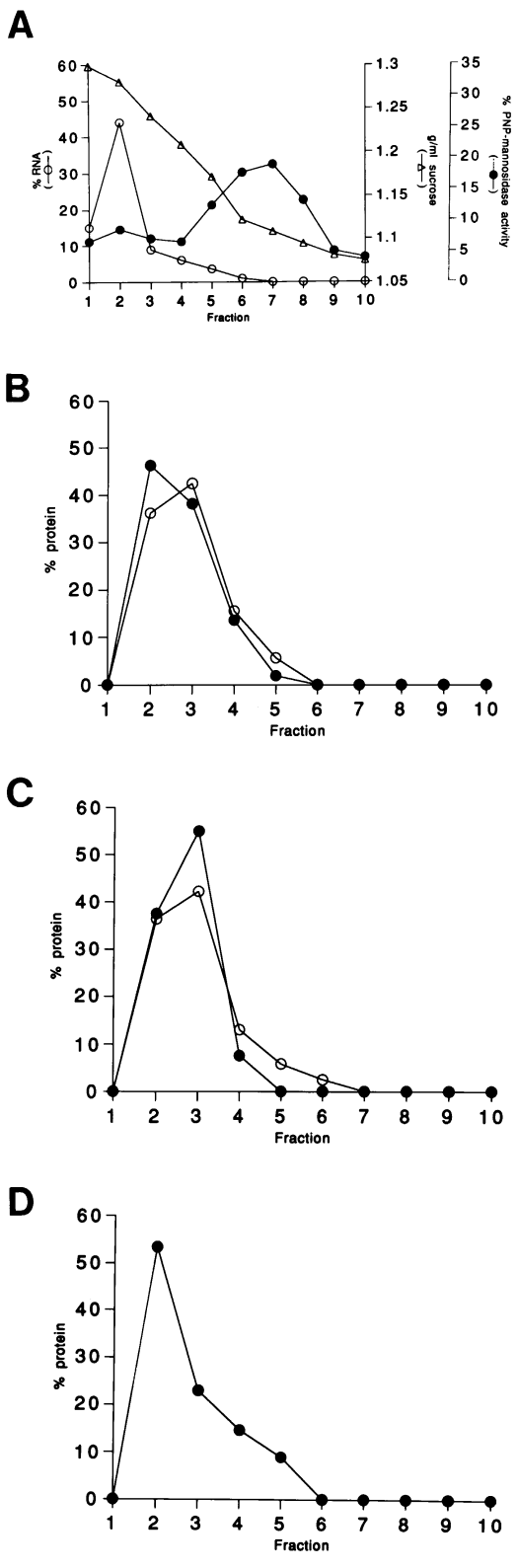

Figure 8 Concentration of intermediates derived from the intracellular degradation of aggrecan precursors in an ER subcompartment

(**A**) Microsomes prepared from labelled chondrocytes (100 μCi/ml for 1 h continuous labelling) were fractionated in a discontinuous sucrose gradient (△) as described in the Materials and methods section. RNA content (○) and *p*-nitrophenyl (PNP)-α-D-mannosidase activity (●) were determined. Fractions were subjected to immunoprecipitation with anti-aggrecan antibody. Immunoprecipitates were processed for SDS/PAGE and fluorography, and the bands were quantified by scanning densitometry. The distribution throughout the gradient of the 370 kDa precursor (○) and the 190 kDa degradation form (●) is shown as percentage of the total amount of each band recovered from the gradient for both control cells (**B**, **C**) and cells chased in the presence of 100 μg/ml cycloheximide for 2 h (**D**). (**C**) corresponds to fractionated microsomes from which ribosomes were previously released by incubation with 25 mM EDTA.

(Figures 7c and 7c′). In order to identify the aggrecan-related molecules contained in the ER subcompartments, radiolabelled chondrocytes were homogenized and fractionated in a discontinuous sucrose gradient; aggrecan-related proteins were then immunoprecipitated from the different subcellular fractions and analysed by SDS/PAGE and fluorography (Figure 8). Fractions 2–4 (specific gravity 1.24–1.17 g/ml) contained both the 190 kDa intermediate and the 370 kDa precursor (Figure 8B). However, when ribosomes were released from the microsomes with EDTA [26], the 190 kDa degradation intermediate was still distributed in the heaviest fractions of the gradient whereas the 370 kDa precursor appeared additionally concentrated in fractions 5 and 6 (Figure 8C). Although only a small fraction (8–10 %) of the 370 kDa precursors was usually displaced in the gradient by EDTA treatment, the change in density was significant (from

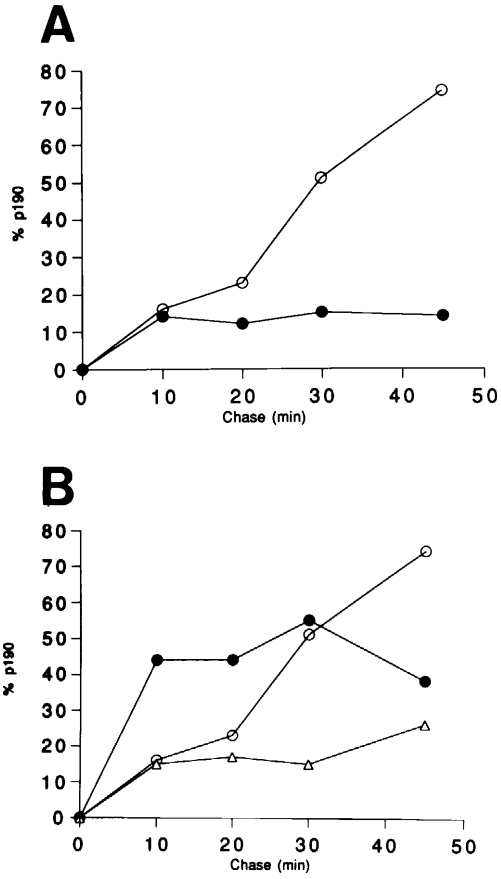

Figure 9 Effect of Ca^{2+} depletion and the redox state on degradation of aggrecan precursors in the ER

Chondrocytes were pulse-labelled for 5 min and chased at 37 °C for the indicated times. (**A**) Cells were chased in unlabelled Ca^{2+}-free medium containing (●) or not (○) 5 μM Ca^{2+} ionophore A23187. (**B**) Cells were chased in regular culture medium containing 1 mM DTT (●), 1 mM diamide (△) or with no addition (○). Aggrecan-related proteins were immunoprecipitated from cell lysates, fractionated by SDS/PAGE, and, after fluorography, the 190 kDa and 370 kDa bands quantified by scanning densitometry. The percentage of the 190 kDa degradation intermediate with respect to the 370 kDa precursor is shown. Data are representative of three different experiments.

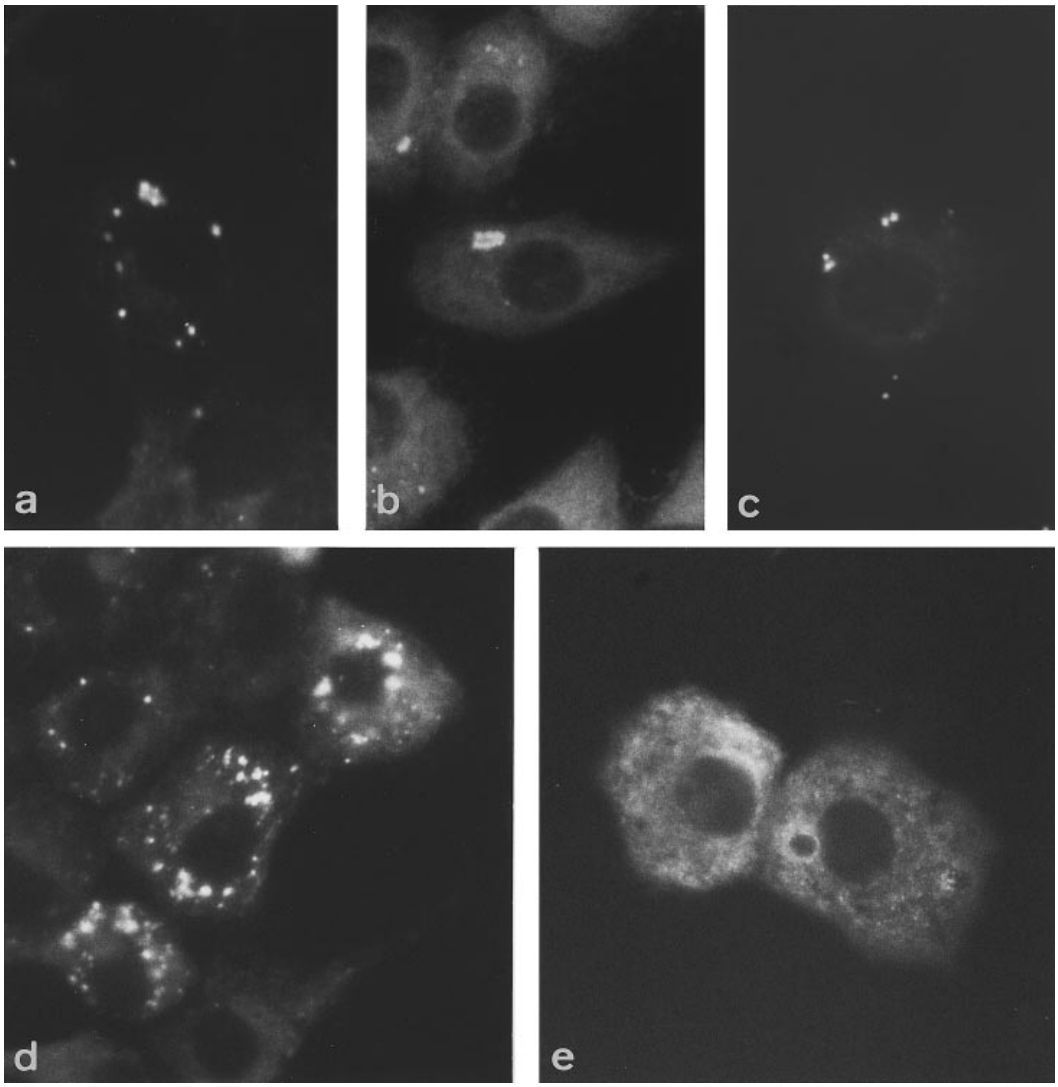

Figure 10 Effect of several treatments on the intracellular localization of aggrecan-related molecules

Cells cultured as monolayers were incubated for 2 h either at 20 °C in regular culture medium (**a**) or at 37 °C in culture medium containing 100 μg/ml cycloheximide (**b**), 200 μg/ml ALLN (**c**), 200 μg/ml leupeptin (**d**) or 2 mM DTT (**e**). After fixation and permeabilization, cells were stained with antibody against aggrecan and processed for immunofluorescence as described in the Materials and methods section. Magnifications: $\times$350.

1.2 g/ml to 1.12 g/ml on average) and limited to this molecular species. This suggested that the degradation intermediate was confined within an organelle of similar buoyant density to, yet distinct from, rough microsomes. In cells chased in the presence of cycloheximide the only intracellular form of aggrecan was the 190 kDa degradation intermediate which remained in the 1.24–1.17 g/ml fractions (Figure 8D). This molecule was localized by immunofluorescence analysis in the ER subcompartments of cycloheximide-treated cells (see Figure 10b). Taken together these results show that the ER subcompartment contained intermediates resulting from degradation of the 370 kDa precursor, and suggest that the degradation event itself occurred in that subregion of the ER.

Factors influencing degradation of aggrecan precursors in the ER

To determine conditions that promote or inhibit degradation of the 370 kDa precursor in the ER we chased the cells under different conditions after a short (5 min) pulse. It has been reported that changes in the Ca^{2+} concentration of the ER lumen interfere with degradation of proteins retained in this organelle [27–29]. As shown in Figure 9(A), incubation of the cells with the Ca^{2+} ionophore A23187 caused a 5-fold decrease in the degradation rate whereas the amount of aggrecan proteoglycans secreted into the medium was increased by about 10% compared with control untreated cells. Likewise, ER degradation of aggrecan precursors was dependent on the redox state of the ER lumen. Degradation of the 370 kDa precursor was stimulated by the reducing agent DTT and it was partly inhibited by diamide, a permeant thiol-oxidizing reagent (Figure 9B). These two agents affected in a similar way proteoglycan secretion as they both caused little retention of aggrecan precursors within cells (33% compared with 26% retention in control cells). In addition, in cells incubated with the alkylating agent *N*-ethylmaleimide, degradation was 70% inhibited (not shown). In keeping with recent reports [28,30,31], these observations suggest that free

thiol groups are needed for optimal ER degradation. They are also consistent with the possible role of serine/cysteine proteases in ER degradation of aggrecan precursors (Figure 4, Table 1). On the other hand, degradation was inhibited by low temperature and was insensitive to energy depletion by NaN_3 and NaF treatment (Table 2). As shown above, cycloheximide did not significantly inhibit the appearance of the 190 kDa degradation intermediate (Figure 8C); but it did inhibit further proteolysis of this molecular form as it remained within the ER degradation subcompartment for long periods (Figure 10b).

Degradation occurs within the ER smooth subcompartment

We took advantage of the above findings showing degradation enhancement or inhibition under different incubation conditions to examine the effects of these treatments on the morphological localization of aggrecan-related molecules by immunofluorescence (Figure 10). Aggrecan-related molecules were clearly detected in fluorescent vesicles, equivalent to ER subcompartments, in cells exposed to conditions that inhibit degradation such as incubation at low temperature (Figure 10a) or with leupeptin (Figure 10d). In fact, we observed a significant increase in the number and size of the vesicles with some of these inhibitory treatments (compare, for example, Figures 10c and 10d), probably resulting from accumulation of the 370 kDa precursor (Figure 4, Table 2). In contrast, ER subcompartments containing aggrecan-related molecules were scanty in cells treated with DTT which, as indicated above, induces a 2-fold increase in the degradation rate (Figure 10e). It can be concluded from these observations that degradation is preceded by transport of the 370 kDa precursor into the smooth ER subcompartment. Thus inhibition of degradation results in accumulation of intact precursors within this compartment.

DISCUSSION

In this study we have examined the intracellular degradation of aggrecan core protein precursors synthesized by cultured chicken chrondrocytes. Our data indicate that, during the processing of the initial 370 kDa aggrecan core protein precursor, a 190 kDa degradation intermediate is normally generated. A 180 kDa fragment was also detected in some experiments but its relationship to the aggrecan core protein molecule could not be unequivocally demonstrated.

Both subcellular fractionation and immunolocalization experiments indicate that degradation of the aggrecan core protein precursors occurs within an ER smooth subcompartment previously described in this cell type [14]. After ribosome release from microsomal membranes, the 190 kDa degradation intermediate was found in a smooth-membrane fraction distinct from those fractions containing exclusively the 370 kDa precursor. In cycloheximide-treated chondrocytes, the membrane fraction containing the degradation intermediate corresponded to the ER subcompartments detected by immunofluorescence in these cells (Figures 8D and 10b). In addition, inhibition of the degradation process induced accumulation of the 370 kDa precursor and this was morphologically noted as an increase in the number and size of the ER subcompartments. The simplest explanation for these observations is that a fraction (estimated to be 25–27%) of aggrecan precursors is continually targeted for degradation into a specialized ER smooth subcompartment. Alternatively, ER degradation of aggrecan precursors is not restricted to a particular subregion in the organelle. This possibility, however, is not supported by our observation that an increase in the degradation rate of aggrecan precursors correlated with a decrease in their presence in ER subcompartments in chondrocytes treated with DTT (Figures 9B and 10e).

Protein degradation is considered to be part of the quality-control system in the ER [12], providing an efficient way of disposing of polypeptides incompetent for transport to the *cis*-Golgi. This system is very selective, since only misfolded proteins or unassembled subunits are degraded [18,32]. Compartmentalization of the degradation reactions decreases the possibility of interference with other events occurring in the ER and provides a means of optimizing degradation reactions. Both the segregation of proteolytic enzymes and their substrates and the control of the ionic and pH conditions in the degradation compartments could favour rapid proteolysis. A specialized subcompartment could also be the site where the cytosolic and ER proteolytic pathways converge [33,34]. Properly folded and assembled proteins could avoid degradation by not being targeted to these compartments. However, other alternatives are also possible. For instance, compartmentalization might be unnecessary if the degradation machinery were highly selective and able to distinguish misfolded proteins from correctly folded molecules.

The factors that determine degradation of some aggrecan precursors in the ER are unknown. One can speculate that the precursors to be degraded are misfolded or underglycosylated proteoglycans actively retained in the ER. In fact, retention of some 370 kDa precursors was noted in previous studies describing aggrecan biosynthesis and processing by cultured chick chondrocytes [35]. In addition, we have observed association of the aggrecan precursors contained in ER subcompartments with the chaperone immunoglobulin-heavy-chain-binding protein (BiP) (M. Alonso and A. Velasco, unpublished work), which is permanently bound to misfolded and incompletely assembled proteins [36]. Alternatively, degradation could result from non-stoicheiometric production of aggrecan precursors and other extracellular matrix proteins. For instance, intracellular interaction between aggrecan core protein and link protein has been proposed to explain the co-ordinated synthesis of these two molecules by chondrocytes [35]. Lack of association with link proteins in the ER could leave some aggrecan precursors unprotected from degradation. A similar situation was recently described in cells overexpressing the major histocompatibility complex class I molecules in the absence of antigenic peptides. Before degradation these proteins become accumulated in an expanded ER subcompartment [13]. It is also possible that an excess of aggrecan precursors forms insoluble aggregates in the lumen of the ER degradation compartment, as is found in cobalt-treated pancreatic cells [5] and thyroxine-treated thyrotrops [37].

The existence of degradative compartments in the ER extends the number of functionally distinct subcompartments and supports the view that the ER is a compartmentalized organelle [1]. Each event in protein biosynthesis, i.e. translation, subunit assembly, glycosylation, degradation, etc., could potentially be localized within a specialized ER subregion. Further investigation is necessary to elucidate the mechanisms by which this compartmentalization is maintained.

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