Precursors of Chondroitin Sulfate Proteoglycan Are Segregated within a Subcompartment of the Chondrocyte Endoplasmic Reticulum

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Abstract. Immunocytochemical methods were used at the levels of light and electron microscopy to examine the intracellular compartments of chondrocytes involved in extracellular matrix biosynthesis. The results of our studies provide morphological evidence for the compartmentalization of secretory proteins in the ER. Precursors of the large chondroitin sulfate proteoglycan (CSPG), the major proteoglycan species produced by chondrocytes, were present in the Golgi complex. In addition, CSPG precursors were localized in specialized regions of the ER. Link protein, a separate gene product which functions to stabilize extracellular aggregates of CSPG monomers with hyaluronic acid, was segregated similarly. In contrast, type II procollagen, another major secretory molecule produced by chondrocytes, was found homogeneously distributed throughout the ER. The CSPG precursor-containing ER compartment exhibits a variable tubulo-vesicular morphology but is invariably recognized as an electronlucent, smooth membrane-bounded region continuous with typical ribosome-studded elements of the rough ER. The observation that this ER structure does not stain with antibodies against resident ER proteins also suggests that the compartment is a specialized region distinct from the main part of the ER. These results support recent studies that consider the ER as a compartmentalized organelle and are discussed in light of the possible implications for proteoglycan biosynthesis and processing.

PROTEINS destined for secretion are transported from the rough ER (rER)[1] to the Golgi and through the Golgi to the extracellular space (Palade, 1975). The polypeptides undergo covalent co- and posttranslational modifications and may become associated with other polypeptides as they continue along their route (Farquhar and Palade, 1981; Farquhar, 1985). Eventually, secreted proteins are sorted from lysosomal, membrane, and even other secretory proteins (Farquhar, 1985; Pfeffer and Rothman, 1987). Although considerable progress has been made in the elucidation of the cellular and molecular bases of these processes, many questions remain concerning the specific sites, sequences of events, and regulatory mechanisms involved in intracellular transport. For example, it is known that the Golgi complex functions as a multicompartment organelle and is involved in the sorting of proteins into lysosomes and in the segregation of secretory products into distinct types of secretory vesicles, but the mechanisms controlling these events are not known (Farquhar, 1985; Burgess and Kelly, 1987; Orci et al., 1987; Pfeffer and Rothman, 1987; Chung et al., 1989). New data indicate that the segregation of molecules may begin earlier in the exocytotic pathway (Munro and Pelham, 1987; Rothman, 1987; Kabcenell and Atkinson, 1986). Differential rates of exit from the ER have been reported, and movement from the ER to the Golgi is frequently the rate-limiting step in the transport of membrane and secretory proteins to the cell surface (Strous and Lodish, 1980; Fitting and Kabat, 1982; Lodish et al., 1983; Fries et al., 1984; Scheele and Tartakoff, 1985; Williams et al., 1985). These observations suggest that processes in the ER may play a role in the regulation of exocytotic membrane trafficking. Other results would suggest that functional subcompartments may be characteristic of the ER as well (reviewed by Rose and Doms, 1988).

A variety of exocrine (Strous and Lodish, 1980; Lodish et al., 1983; Fries et al., 1984; Scheele and Tartakoff, 1985; Saraste et al., 1986) and endocrine (Green and Shields, 1984; Orci et al., 1987; Chung et al., 1989) cell systems are being used to investigate the movement of proteins through the exocytotic pathway. Studies using viruses and their glycoproteins have been particularly informative (reviewed by Pfeffer and Rothman, 1987; Rose and Doms, 1988). Other processes amenable to studies of intracellular trafficking include those involved in the synthesis and assembly of extracellular matrix (ECM) molecules. Cells that elaborate ECM are dedicated to the production of specific, well-characterized proteins, often in large quantities. ECM molecules undergo numerous processing events and may participate in a variety of macromolecular interactions as they move through cytoplasmic membrane compartments (reviewed by

1. *Abbreviations used in this paper*: CSPG, chondroitin sulfate proteoglycan; ECM, extracellular matrix; rER, rough endoplasmic reticulum.

Hay, 1981). We have used cartilage cells in culture as a model system for the study of protein trafficking. Chondrocytes are committed to the synthesis, processing, and assembly of an ECM composed of abundant amounts of two major macromolecules, type II collagen, and chondroitin sulfate proteoglycan (CSPG). Large aggregates of CSPG are formed extracellularly by the link protein-stabilized association of CSPG monomer and hyaluronic acid. The CSPG monomer itself (M_r 1–5 × 10^6) has a 300–350-K core protein with many covalently attached chondroitin sulfate and keratan sulfate glycosaminoglycan chains, N-linked oligosaccharides, and O-linked oligosaccharides (Hascall, 1981; Heinegård and Paulsson, 1984; Hassell et al., 1986; Lohmander and Kimura, 1986; Carney and Muir, 1988; Ruoslahti, 1988). Link protein is also characterized by the addition and further modification of N-linked oligosaccharides. In this case, multiple forms of link protein are generated by differential glycosylation and sulfation (Caterson et al., 1985; Choi et al., 1985; Hering and Sandell, 1988, manuscript submitted for publication). The biosynthetic modifications of type II collagen involve the translation of type II procollagen, hydroxylation of proline and lysine, glycosylation of asparagine and hydroxylysine, formation of interchain disulfide bonds, and assembly of triple helical molecules. The enzymatic conversion of procollagen to collagen and collagen fiber formation occur extracellularly (Prockop et al., 1979; Olsen, 1981; Kivirikko and Myllyla, 1984).

Intracellular sites for the synthesis, processing, and assembly of cartilage ECM molecules have been investigated. Ultrastructural studies have characterized the extensive rER and Golgi complex of chondrocytes (Thyberg et al., 1973; Hascall, 1980; Takagi et al., 1981). Functional properties of chondrocyte organelles have been determined using autoradiography (Revel and Hay, 1963; Fewer et al., 1964; Godman and Lane, 1964; Kimata et al., 1971), subcellular fractionation (Fellini et al., 1981; Geetha-Habib et al., 1984; Campbell and Schwartz, 1988), and immunohistochemistry (Vertel and Dorfman, 1979; Vertel and Barkman, 1984; Pacifici et al., 1984; Ratcliffe et al., 1985; Vertel et al., 1985*a*,*b*; Velasco et al., 1988). As in other cell types, the cotranslational addition of N asparagine-linked, high-mannose oligosaccharides occurs in the rER, while the Golgi complex is the site for the further modification of N-linked oligosaccharides and for the elongation and sulfation of glycosaminoglycan chains (Geetha-Habib et al., 1984; Kimura et al., 1984; Lohmander et al., 1986; Vertel and Hitti, 1987). While we can assign intracellular locations for many processing events, others remain obscure. For example, the subcellular site for xylosylation of the CSPG core protein before glycosaminoglycan chain elongation is not resolved (Geetha-Habib et al., 1984; Hoffmann et al., 1984; Lohmander et al., 1986; Nuwayhid et al., 1986), and it has been proposed that the CSPG core protein and link protein associate before secretion (Kimura et al., 1981; Vertel and Barkman, 1984; Campbell and Schwartz, 1988), but neither an assembly event, nor a site of assembly has been demonstrated.

In previous studies we investigated intracellular features of the biosynthesis and processing of cartilage matrix molecules. Double immunofluorescence localization experiments revealed the segregation of type II procollagen and CSPG precursors within the cytoplasm of cultured chondrocytes (Vertel and Barkman, 1984; Vertel et al., 1985*a*,*b*). In the present study we use immunoelectron microscopy to demonstrate that this heterogeneity exists within the ER of chondrocytes, providing morphological evidence for the sorting and segregation of newly synthesized molecules in the ER. These results suggest that segregation within the ER can occur during the normal course of synthesis and processing of ECM molecules and are discussed in the context of subcompartmentalization of the ER.

Materials and Methods

Materials

Fertile White Leghorn chicken eggs were purchased from Cornell University (Ithaca, NY) and Sharp Farms (Glen Ellyn, IL). Trypsin, Ham's F-12 medium, FCS, antibiotic-antimycotic mixture, Hank's balanced salt solution (HBSS), and goat serum were obtained from Grand Island Biological Supply Co. (Grand Island, NY). Testicular hyaluronidase was a product of Leo (Helsingborg, Sweden). Goat anti-guinea pig IgG, goat anti-rabbit IgG, and goat anti-mouse IgG coupled to FITC or TRITC, and Fab fragments of goat anti-rabbit IgG coupled to horseradish peroxidase were obtained from Cappel Laboratories (Malvern, PA). Saponin, sodium borohydride, diaminobenzidine, potassium ferrocyanide, polyethylene glycol, sodium *m*-periodate, and BSA were products of Sigma Chemical Co. (St. Louis, MO). Glutaraldehyde, osmium tetroxide, hydroxypropyl methacrylate, and Epon were purchased from Electron Microscopy Sciences (Fort Washington, PA); Lowicryl and paraformaldehyde were from Polysciences, Inc. (Warrington, PA); tannic acid was from Mallinckrodt (Paris, KY), protein A was from Pharmacia Fine Chemicals (Uppsala, Sweden); and tetrachloroauric acid was from Fisher Scientific (Pittsburgh, PA).

Cell Culture

Cartilage cells were prepared from the sterna of 15-d-old chicken embryos as described by Cahn et al. (1967). For immunofluorescence studies, cells were cultured in monolayer on gelatinized, carbon-coated coverslips at a density of 4 × 10^5 cells per 60-mm tissue culture dish in 3 ml Ham's F-12 medium containing 10% FCS and 1% antibiotic-antimycotic mix. For electron microscopy, cells were cultured onto gelatinized 35-mm tissue culture dishes at the same density in 1.5 ml of the same medium. Cell cultures were fed with fresh medium on days 2 and 3 and fed again several hours before fixation on day 4.

Immunofluorescent Staining

Cells on coverslips were incubated with testicular hyaluronidase for 10 min at 37°C to remove extracellular CSPG before fixation, as described previously (Vertel and Dorfman, 1979). After hyaluronidase digestion, cells were rinsed several times with HBSS and fixed with 75% ethanol. Coverslips with fixed cells were rinsed with 75% ethanol, treated for 2 min with 98% ethanol/ether (1:1 [vol/vol]), and air dried (von der Mark et al., 1977).

Permeabilized cells were incubated in primary antibodies for 20 min at room temperature, washed with HBSS, and incubated for 20 min with goat anti-rabbit, goat anti-guinea pig, or goat anti-mouse IgG coupled to either FITC or TRITC. After further washes, coverslips were mounted in phosphate buffer/glycerol (1:9, [vol/vol]). Samples were observed and photographed using a Leitz Ortholux microscope with phase and epifluorescence optics. Fields were selected from double-stained specimens and photographed sequentially for FITC and TRITC staining.

Antibodies

Polyclonal rabbit and guinea pig antibodies directed against (*a*) hyaluronidase-digested CSPG monomer, and (*b*) pepsin-extracted type II collagen from chicken cartilage were prepared and characterized previously (Vertel and Dorfman, 1979; Upholt et al., 1979). Antibody specificity was determined by radioimmunoassay (Vertel and Dorfman, 1979), immunoprecipitation (Upholt et al., 1979; Vertel and Hitti, 1987; O'Donnell et al., 1988), and immunoblot analysis. The antibodies were shown to immunoprecipitate cell-free translated products as well as intracellular and extracellular forms of these matrix molecules. Immunofluorescence staining of intracellular compartments and the ECM using these antibodies have been reported (Ver-

tel and Dorfman, 1979; Vertel and Barkman, 1984; Vertel et al., 1985*a*,*b*). The polyclonal antibodies for CSPG monomer exhibit the same characteristics of immunoreactivity as the rat monoclonal antibody S103L, a monoclonal antibody that recognizes an epitope of the CSPG core protein (Upholt et al., 1981). Thus, both the monoclonal and polyclonal antibodies to the CSPG monomer react independently of posttranslational modifications; they react well with unmodified, cell-free translated core protein, biosynthetic intermediates, and extracellular CSPG in aggregate. Type II collagen antibodies recognize cell-free translated type II (pre)procollagen, intracellular procollagen, and extracellular, fibrillar collagen. Although our type II collagen antibodies cannot distinguish procollagen from collagen on the basis of immunoreactivity, others have established that the conversion of procollagen to collagen is an extracellular process (Prokop et al., 1979; Olsen, 1981); therefore, we refer to all intracellular forms of collagen as procollagen. Guinea pig antibodies to cartilage link protein recognize both intracellular and extracellular forms of the protein and were described previously (Vertel et al., 1985*b*). These antibodies were characterized by radioimmunoassay, immunoprecipitation, immunoblot analysis, and immunofluorescence reactivity. The 5-D-4 mouse monoclonal antibody obtained from Dr. Bruce Caterson (West Virginia Medical Center, Morgantown, WV) recognizes keratan sulfate glycosaminoglycans (Caterson et al., 1983). Polyclonal antibodies against resident proteins of the rER provided by Dr. Daniel Louvard (Institute Pasteur, Paris) were described by Louvard et al. (1982). For immunolocalization reactions, purified IgG (for anti-CSPG) or antisera were used at dilutions varying from 1:10 to 1:100. Ascites fluid for the 5-D-4 monoclonal antibody was used at a dilution of 1:50.

Immunoperoxidase Staining and Electron Microscopy

The procedure described by Brown and Farquhar (1984) was used with some modifications. Chondrocyte cultures were digested with hyaluronidase as described, washed with HBSS, and fixed at 25°C with either 2% paraformaldehyde/0.75% lysine/10 mM $NaIO_4$/35 mM phosphate buffer, pH 6.2 for 2 h (McLean and Nakane, 1974) or with 0.15% glutaraldehyde/HBSS for 10 min. Cells were rinsed several times with PBS (0.15 M NaCl in 0.01 M phosphate buffer, pH 7.4), and those fixed with glutaraldehyde were treated with 1% sodium borohydride for 30 min to quench unreacted aldehyde groups and restore antigenicity lost by glutaraldehyde fixation (Eldred et al., 1983). Permeabilization was accomplished by incubation in 0.01% saponin/5% goat serum/PBS (buffer A) for 30 min at room temperature. Cells were incubated for 60 min at 37°C in primary antibodies (1:5 to 1:20 dilution in buffer A) or in normal rabbit serum as a control. Samples were washed for 30 min with buffer A and incubated for 60 min at 37°C in horseradish peroxidase-coupled goat anti-rabbit IgG Fab fragments (diluted 1:75 in buffer A). After rinses in buffer A, the samples were fixed for 60 min in 1.5% glutaraldehyde/0.1 M Na cacodylate, pH 7.4/5% sucrose, washed sequentially with 0.1 M Na cacodylate, pH 7.4/5% sucrose and 0.05 M Tris-HCl, pH 7.4/7.5% sucrose, and incubated for 5 min in 0.2% diaminobenzidine/0.05 M Tris-HCl, pH 7.4/7.5% sucrose. Horseradish peroxidase-linked antibody products were visualized by a 10-min incubation in 0.2% diaminobenzidine/0.05 M Tris-HCl, pH 7.4/7.5% sucrose, containing 0.01% H_2O_2. Subsequently, cells were washed in 0.05 M Tris-HCl, pH 7.4/7.5% sucrose, and post-fixed for 1 h at 4°C in 1% OsO_4/1.5%$[K_4Fe(CN)_6]$ in 0.1 M Na cacodylate, pH 7.4. Dehydration through a series of ethanol solutions and through a graded series of hydroxypropyl methacrylate and Epon 812 solutions and embedment in Epon 812 were accomplished according to the procedure of Brinkley et al. (1967). Sections were cut parallel to the plane of the cell monolayers, and observed in a JEOL CX100 or a Zeiss EM10 transmission electron microscope, usually after counterstaining with lead citrate for 3 min.

Embedment in Lowicryl and Protein A–Colloidal Gold Localization

Hyaluronidase-digested cultures were fixed in 0.5% glutaraldehyde/PBS for 1 h at room temperature. Cells were rinsed with several changes of PBS and incubated in 50 mM NH_4Cl/PBS for 1 h at room temperature. After additional PBS washes, cells were scraped from the culture dishes and embedded as pellets in Lowicryl K4M resin (Roth et al., 1981). Ultrathin sections were collected onto Parlodion carbon-coated nickel grids. Grids were floated on 1% BSA in PBS before incubation with primary antibodies (1:10 dilution in BSA/PBS) for either 2 h at room temperature or 12 h at 4°C. After two 5-min washes with PBS and one with BSA/PBS, grids were incubated for 1 h at room temperature with protein A-gold complexes prepared according to the procedure of Slot and Gueze (1985) using a monodisperse solution of 8-nm gold particles. Protein A–gold complexes were used at a concentration of $Absorbance_{525\ nm} = 0.06$. After two 5-min rinses with PBS and double-distilled water, grids were counterstained with aqueous uranyl acetate for 5 min and with lead citrate for 2 min before observation in the electron microscope.

Results

A Distinct CSPG Precursor-containing Compartment Is Present in Chondrocytes

The intracellular distribution of CSPG and its precursors was characterized in cultured chondrocytes using simultaneous double immunofluorescence localization reactions. As shown in Fig. 1, CSPG monomer and its precursors are localized in perinuclear crescents (*arrows*) and in peripheral cytoplasmic compartments (*arrowheads*). Only the perinuclear crescents react positively with antibodies that recognize keratan sulfate glycosaminoglycans (Fig. 1 *c*). In addition, the perinuclear crescents, but not the peripheral vesicles, react with fluorescent-labeled ricinus and wheat germ agglutinin, two lectins that recognize carbohydrates added in the Golgi complex (not shown, but see Vertel et al., 1985*a*,*b*). Thus, based on antibody and lectin reactivities (and on the ultrastructural analysis discussed below), the perinuclear crescents correspond to the sites of Golgi complexes and contain processed forms of CSPG molecules. The immunoreactive compartments outside of the perinuclear regions contain CSPG precursors which apparently have not been modified by Golgi-mediated processes.

It is noteworthy that type II procollagen is localized in regions throughout the chondrocyte cytoplasm, but seems to be excluded from the CSPG precursor-containing compartments (Fig. 2, *a* and *b*). In contrast, link protein is colocalized in CSPG precursor-containing regions (Fig. 2, *c* and *d*). (Link protein is a constituent of the cartilage ECM that interacts with CSPG monomer in the matrix to stabilize its aggregation with hyaluronic acid.) Thus, based on the analysis of double immunofluorescence localization reactions, both CSPG monomer and link protein precursors are segregated from type II procollagen in the cartilage cell cytoplasm.

The CSPG Precursor-containing Compartment Is a Subcompartment of the Endoplasmic Reticulum

The analysis of immunoperoxidase reactions was undertaken to characterize ultrastructural features of CSPG precursor-containing compartments. As expected, antibodies to CSPG monomer and its precursors were localized within the perinuclear Golgi complex (Fig. 3 *a*). Immunostaining of secretory vesicles and *trans*-Golgi cisternae was noted (Fig. 3 *b*). Some parts of the ER were heavily stained as well (Fig. 3 *a*). Immunoreactive ER structures included terminal, vesicular dilations of typical ER cisternae (Fig. 3 *c*) and branching, tubular structures (Fig. 3, *d* and *e*). These regions were enclosed by smooth rather than ribosome-studded membranes (Fig. 4). In some thin sections of individual chondrocytes, immunoreactive product was concentrated in two or three elaborate, tubulo-vesicular structures (Fig. 3 *f*). Each one of these appeared to be formed by several tubules converging into a common, central area. Independent of the specific morphological features of the CSPG precursor-containing structures, however, their smooth membrane boundary and

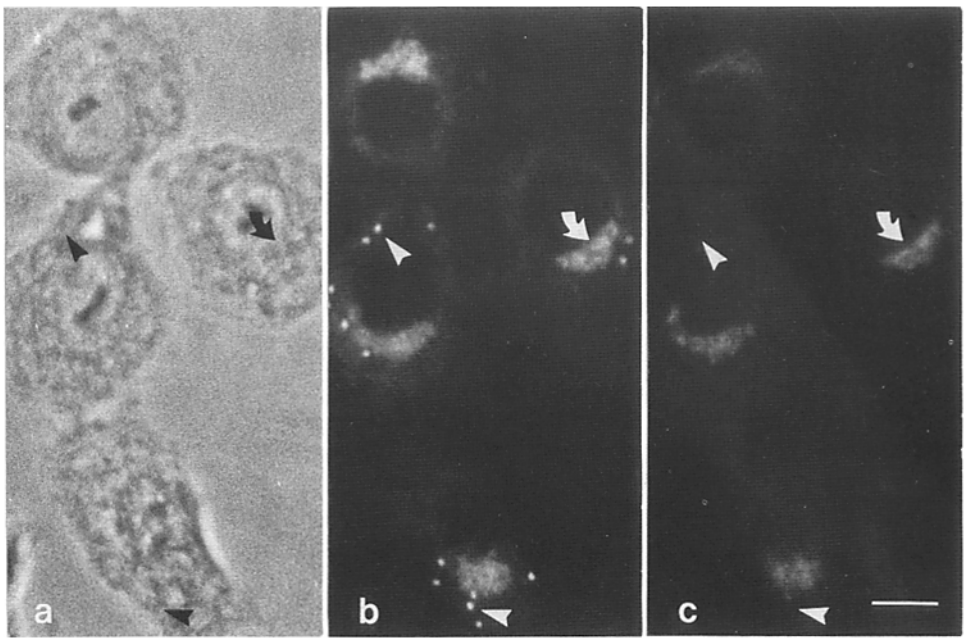

Figure 1. Cytoplasmic compartments of chondrocytes containing CSPG and precursors. Chondrocytes on coverslips were prepared as described in Materials and Methods and incubated with antibodies in the following order: mouse monoclonal 5-D-4 antibody (which recognizes keratan sulfate glycosaminoglycans); TRITC-coupled goat IgG anti-mouse IgG; guinea pig polyclonal anti-CSPG; and FITC-coupled goat IgG anti-guinea pig IgG. The same field of double-stained cells is shown under phase to the left (*a*). The perinuclear crescents (*arrows*) stain with both CSPG polyclonal antibodies (*b*) and KS monoclonal antibodies (*c*). CSPG precursor-containing vesicles (*arrowheads*) stain with antibodies that recognize CSPG precursors (*b*), but do not stain with antibodies that recognize keratan sulfate glycosaminoglycans (*c*). Bar, 2 μm.

continuity with typical elements of the rER were observed regularly. As shown in Fig. 4, the immunoreactive tubules are continuous with expanded regions of the rER. Although staining was not absent completely from other parts of the ER (e.g., Fig. 3 *f*), it was considerably more intense in the specialized structures just described. Thus, the intensely stained ER regions should be considered the ultrastructural counterparts of the peripheral vesicles demonstrated at the level of light microscopy by immunofluorescence. The continuity of these specialized regions with the rER is evident from the adjacent sections shown in Fig. 5. The presence of occasional vesicular buds at the surface of these structures may reflect the transport of product to or from these ER compartments.

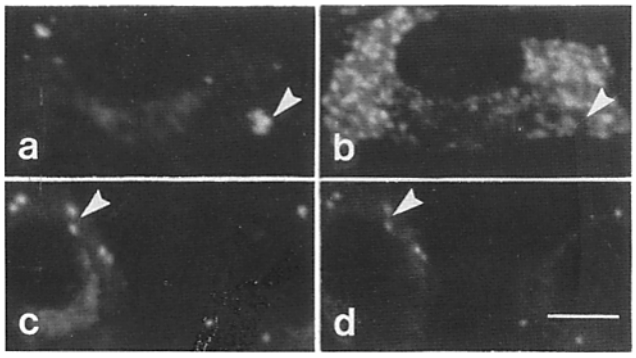

Figure 2. Cytoplasmic structures containing precursors of CSPG monomer exclude type II procollagen but contain link protein. Chondrocytes shown in *a* and *b* were incubated simultaneously in guinea pig anti-CSPG and rabbit anti-type II collagen followed by simultaneous incubation in FITC-coupled goat IgG anti-guinea pig IgG and TRITC-coupled goat IgG anti-rabbit IgG. Staining of CSPG and its precursors is shown to the left (*a*) and type II collagen staining is shown to the right (*b*) for the same field of double-stained cells. Note that the CSPG precursor-containing structures (*arrowheads*) do not stain with type II collagen antibodies. Chondrocytes shown in *c* and *d* were incubated with antibodies in the following order: (*a*) guinea pig anti-link protein; (*b*) TRITC-coupled goat IgG anti-guinea pig IgG; (*c*) rabbit anti-CSPG; (*d*) FITC-coupled goat IgG anti-rabbit IgG. Staining of CSPG and its precursors is shown to the left (*c*), and staining for link protein is shown to the right (*d*). Vesicles (*arrowheads*) are reactive with antibodies to both CSPG and link protein, demonstrating that the precursors of CSPG aggregates are colocalized in cytoplasmic structures that do not stain with type II collagen antibodies. Bar, 2 μm.

A similar staining pattern was observed when the intracellular localization of link protein was investigated using immunoperoxidase reactions. The reaction product was often concentrated in restricted regions of the ER (Fig. 6 *a*). In contrast, type II collagen antibodies were localized in a homogeneous pattern throughout the main lumen of the rER (Fig. 6 *b*). Thus, type II collagen staining of long cisternae and expanded regions of the ER was observed.

As described above, a clear correlation exists between immunofluorescence staining patterns and the ultrastructural localization patterns determined from immunoperoxidase reactions for CSPG monomer, link protein, and type II collagen precursors. However, to address the possibility that the differential staining of restricted regions of the ER was the result of an artifact of incomplete penetration of immunoreagents, we used a postembedment immunolocalization procedure. As shown in Fig. 7, the concentration of CSPG precursors in specialized ER regions was evident after exposure of the whole surface of ultrathin sections of Lowicryl-embedded chondrocytes to CSPG antibodies and protein A–colloidal gold complexes. The morphology of CSPG precursor-containing ER compartments in Lowicryl-embedded chondrocytes was similar to that of ER compartments observed after preembedment immunoperoxidase localization. That is, both vesicular profiles (Fig. 7 *b*) and convergent tubular structures (Fig. 7 *c*) were immunoreactive. In addi-

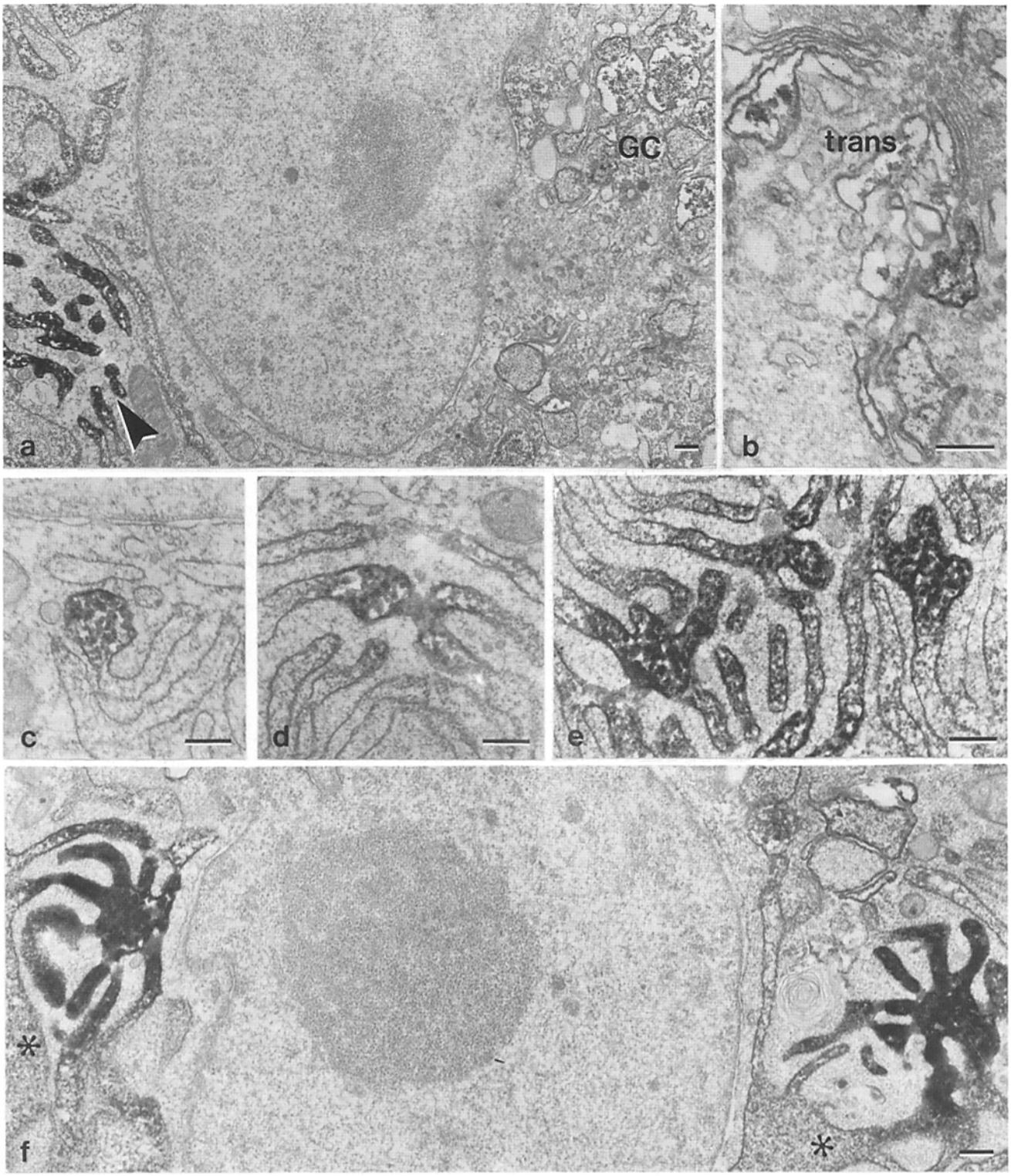

Figure 3. Ultrastructural localization of CSPG precursors in cytoplasmic compartments of chondrocytes by immunoperoxidase. Chondrocytes were fixed with paraformaldehyde-lysine-periodate, and used for immunoperoxidase localization as described in Materials and Methods. (*a*) The general immunoreaction includes staining of the Golgi complex (*GC*) and several structures associated with the rER (*arrow*). In this figure, the rER-associated profiles are tubular structures. Commonly, reactive tubules are located in cytoplasmic regions distant from the Golgi complex. (*b*) In the Golgi area, secretory vesicles and *trans* cisternae of the Golgi stacks are stained. The *trans* face of the Golgi stacks is indicated. (*c–e*) Regions of the ER that contain concentrated CSPG precursors vary in morphology and in the intensity of staining. Thus, CSPG precursors are segregated in vesicular ends of typical ER cisternae (*c*), in ER vesicles and tubules located in close proximity to each other (*d*), or in complex, branching tubular networks (*e*). (*f*) More elaborate, intensely staining, tubulo-vesicular structures of the ER are often observed. Dilated and cisternal regions of the ER continuous with these structures (*asterisks*) may exhibit a low level of immunoreactivity. Bar, 0.25 μm.

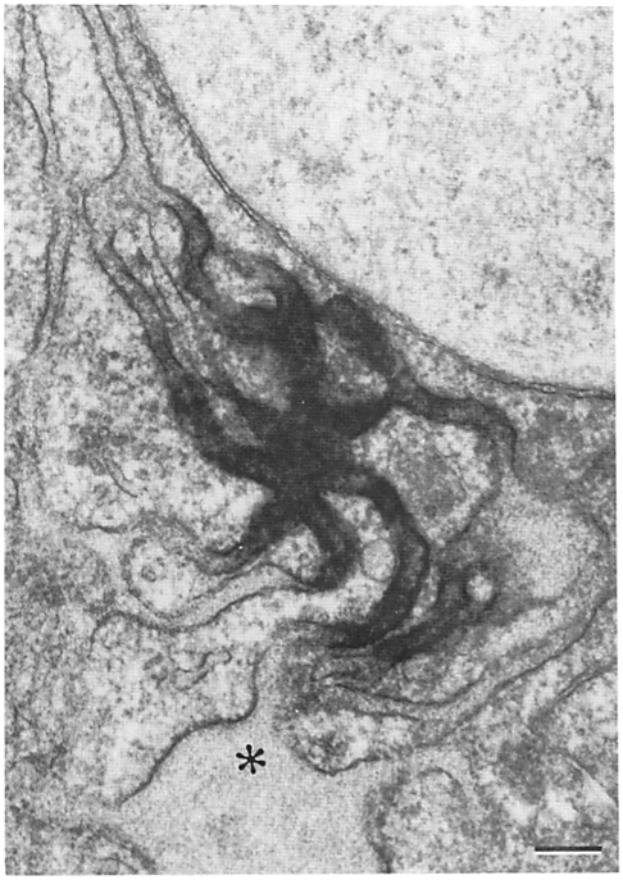

Figure 4. CSPG precursor-containing regions of the ER are smooth membrane-bound and continuous with the ER. A convergent tubular structure containing CSPG precursors is shown in this glutaraldehyde-fixed chondrocyte. The tubules are enclosed by smooth membrane, but are continuous with dilated or cisternal regions of the ER that are studded with ribosomes (*asterisk*). Bar, 0.25 μm.

tion, using this postembedment technique, we noted that the CSPG precursor–containing compartments connected to the rER are electronlucent.

The characteristics of the CSPG precursor–containing regions of the ER were explored further using antibodies to resident proteins of the rER in immunofluorescence localization reactions. As shown, the CSPG precursor-containing structures of the ER do not react with the rER antibodies (Fig. 8, *a* and *b*). Thus, the rER is defined by the localization of rER antibodies and the distribution of intracellular type II procollagen. The double immunolocalization studies demonstrate that the CSPG precursor–containing vesicles of the ER are distinct from the rER.

Discussion

In this study, we demonstrate heterogeneity of the chondrocyte ER by immunofluorescence localization and by both pre- and postembedment immunoelectron microscopy. The segregation of CSPG precursors is observed in regions of the ER that apparently exclude type II procollagen. These regions are continuous with the rER but are characterized by their own unique features. They are smooth membrane–enclosed structures that appear as terminal, vesicular dilations of typical rER cisternae or as branching, tubular structures. Occasional vesicular buds are observed at the surface of these structures. Antibodies directed against resident proteins of the rER do not stain the CSPG precursor–containing structures, supporting the contention that these ER regions represent a distinct subcompartment of the ER.

Compartmentalization is a characteristic feature of the exocytotic pathway. It is well-established that the Golgi complex plays a pivotal role in this pathway, and generally accepted that the Golgi complex is a multicompartment organelle, with distinct functions performed by each subcompartment (Farquhar, 1985). More recently it has been suggested that the ER may be compartmentalized as well (Rose and Doms, 1988). This hypothesis is based on the notion that different membrane and/or secretory proteins are segregated into ER subcompartments where specific synthetic or processing events occur. For example, membrane domains of the ER have been defined. Specialized regions involved in ribosome attachment are rich in ribophorin I and II (Kriebach et al., 1978) and in other proteins that participate in the translocation of the nascent polypeptide across the rER membrane, such as the docking protein or signal recognition protein receptor (Walter and Lingappa, 1986). Other domains include the nuclear envelope, and those areas characterized by the unusual structural organization of the partly rough, partly smooth transitional elements of the ER from which transport vesicles form (Palade, 1975). Special ER compartments have been proposed for the concentration (Saraste and Kuismanen, 1984; Copeland et al., 1988), fatty acylation (Berger and Schmidt, 1985), oligomerization (Bole et al., 1986; Gething et al., 1986; Kreis and Lodish, 1986; Copeland et al., 1988), oligosaccharide processing (Kabcenell and Atkinson, 1985; Tooze et al., 1988), and degradation (Chen et al., 1988; Lippincott-Schwartz, 1988) of protein precursors,

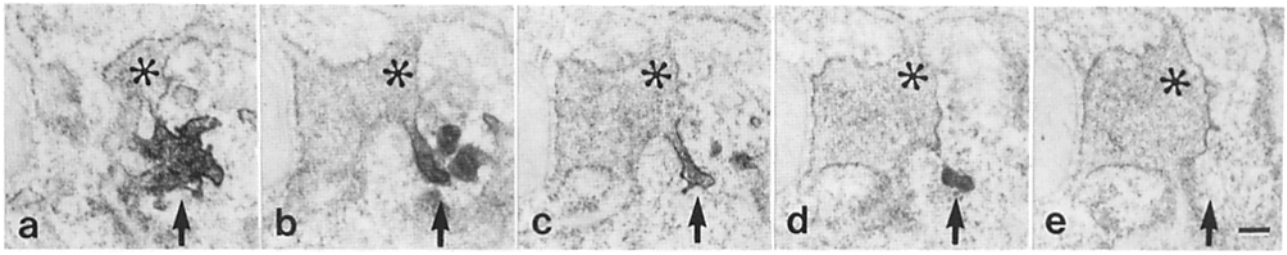

Figure 5. Adjacent sections of a rER-associated, CSPG precursor-containing ER region. Noncounterstained, consecutive sections of a CSPG precursor-containing region continuous with the rER are shown. The same position in the rER lumen (*asterisk*) and the position of the CSPG precursor–containing region (*arrow*) are indicated in each micrograph. Note the vesicular buds along the periphery of the immunoreactive compartment. Bar, 0.25 μm.

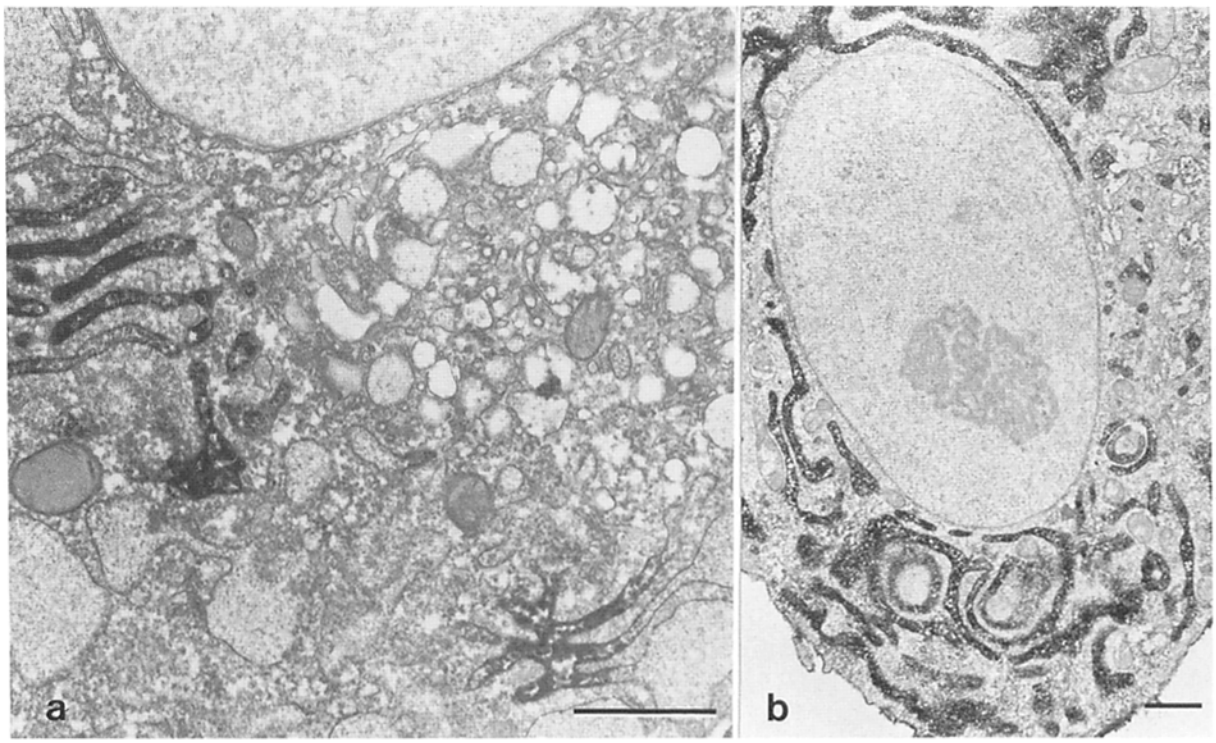

Figure 6. Ultrastructural localization of link protein and type II procollagen. The distribution of link protein (*a*) and type II procollagen (*b*) is shown in the chondrocyte cytoplasm. Link protein staining (*a*) is observed in restricted regions of the ER similar to those reactive with CSPG antibodies. In contrast, type II procollagen (*b*) is distributed homogeneously throughout most of the rER lumen. Bar, 1 μm.

but evidence for this compartmentalization is indirect. In general, the lumenal contents of the ER are considered to be homogeneous in distribution.

Morphological evidence presented in this report demonstrates that segregation exists in the lumen of the ER for at least some secretory proteins. Thus, precursors of two major cartilage matrix components, CSPG monomer and link protein, were found to be co-concentrated in restricted lumenal regions of the chondrocyte ER. Since the observation was made for normal, cultured chondrocytes, segregation in the ER may be part of a common sequence of events involved in the synthesis and secretion of ECM constituents. It is unlikely that these findings are based on artifact since consistent results were obtained using two different immunoelectron microscopic methods. Furthermore, the ultrastructural data correlate with immunofluorescence localization analyses that also indicate the co-concentration of CSPG monomer and link protein precursors in a pre-Golgi compartment.

The CSPG precursor-containing compartment exhibits a variable, vesicular-tubular morphology in the electron microscope. Despite this variation, the structures are uniformly bounded by smooth membrane and continuous with typical ribosome-studded regions of the ER. In this respect, morphological features of these regions and the transitional ER bear some similarity. The transitional ER was initially described as a network of smooth, ribosome-free, vesicular-tubular structures believed to be sites for the formation of transport vesicles (Palade, 1975), often, but not always, in the vicinity of the *cis*-Golgi (see Tooze et al., 1988 for a discussion about the structure and location of the transitional elements). Vesicular buds observed along the smooth membrane-enclosed surface of the CSPG precursor-containing ER structures are also compatible with a transport function. These properties suggest that the subcompartment of the ER we describe may serve to collect or concentrate CSPG precursors before their exit from the ER. However, the relationship, if any, between the CSPG precursor-containing compartment and the transitional ER is not clear since occasional immunostaining was also detected in more typical, transitional ER cisternae on the *cis* side of the Golgi complex (not shown).

Additional possibilities for function, other than the collection or concentration of molecules for transport, may be considered for these CSPG precursor-containing structures. Several lines of investigation indicate that a smooth membrane-enclosed, late ER subcompartment similar to transitional ER functions in carbohydrate processing. Based on studies of energy requirements for α-mannosidase processing of the $Man_8GlcNAc_2$ oligosaccharide of the SA11 rotavirus VP7 glycoprotein, Kabcenell and Atkinson (1985) suggested that oligosaccharide trimming may be a feature of a late ER compartment. Tooze et al. (1988) reported the addition of *N*-acetylgalactosamine to the E1 glycoprotein of mouse hepatitis virus A-59 (an O-linked oligosaccharide modification of serine and threonine residues) in a smooth membrane-enclosed budding region associated with transition vesicles of the ER. Studies of mutant low density lipoprotein receptors by Pathak et al. (1988) also suggest the attachment of O-linked sugars in the rER or the transitional zone of the ER. The subcellular site for xylosylation of the CSPG core protein, a modification involving O-linkage to serine residues, is unresolved. Some investigators have ascribed this function

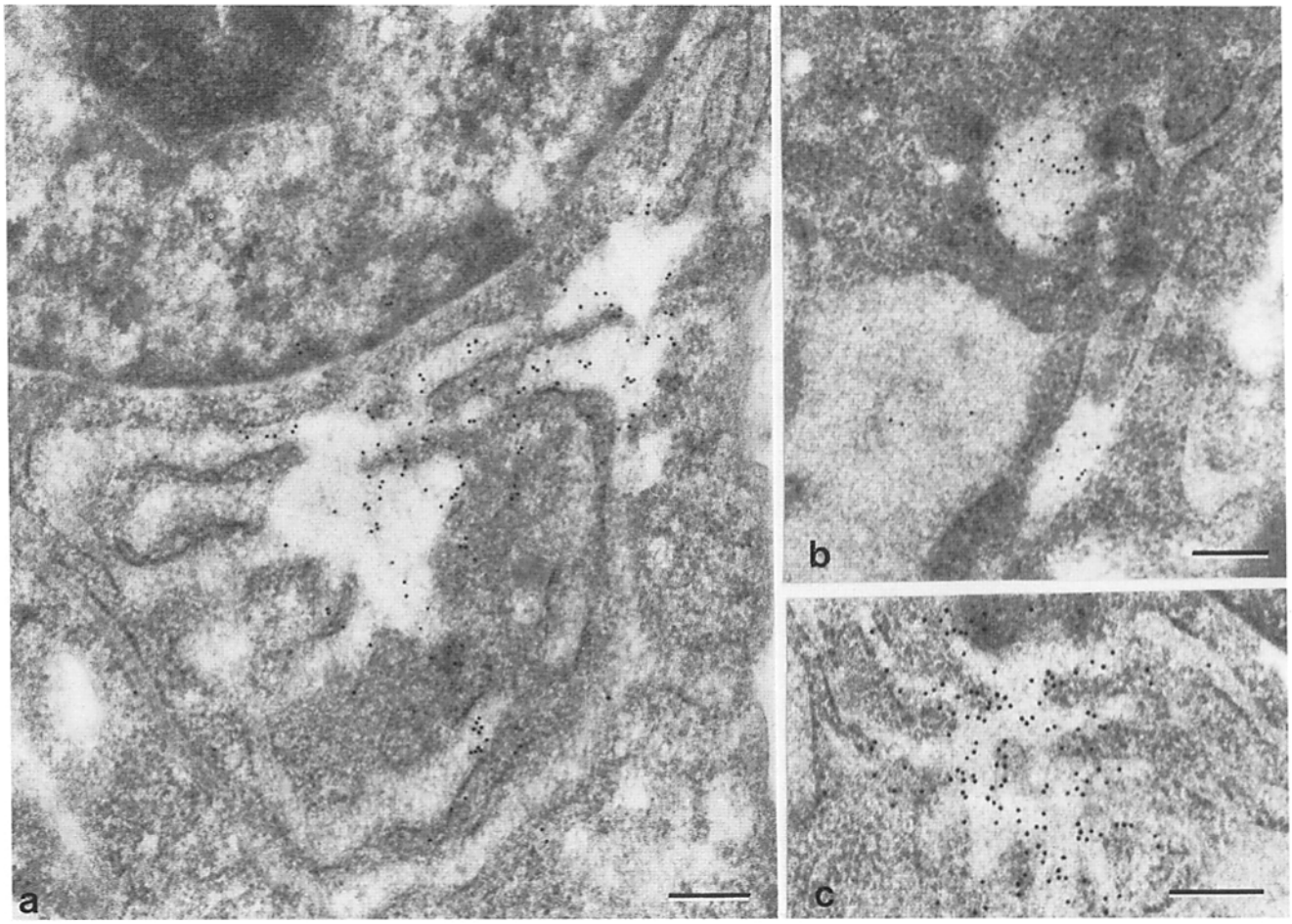

Figure 7. Segregated CSPG precursors are detected by post-embedment immunogold localization. In Lowicryl-embedded chondrocytes, the CSPG precursor-containing compartment appears as an electronlucent, smooth membrane-bound area continuous with typical ribosome-studded regions (*a*). Terminal dilations (*b*) and convergent tubular structures (*c*) are recognized, as in immunoperoxidase-stained chondrocytes. Bar, 0.25 μm.

to the ER (Geetha-Habib et al., 1984; Hoffmann et al., 1984), while others suggest xylosylation occurs in the Golgi complex (Kimura et al., 1984; Lohmander et al., 1986; Nuwayhid et al., 1986). It may be possible that the ER subcompartment of chondrocytes functions in xylosylation.

Alternatively, since CSPG precursors and link protein are relatively concentrated in these regions, and type II procollagen is excluded, perhaps the ER subcompartment represents a site for the achievement of necessary conformational modifications or the association of CSPG precursors before further processing in the Golgi complex. The intracellular association between link protein and CSPG core protein has been suggested previously on the basis of biochemical studies (Kimura et al., 1981; Campbell and Schwartz, 1988), but no data are available concerning the intracellular folding or assembly of CSPG monomer or link protein precursors, or complexes between the two. Whether or not the multiple forms of link protein generated by differential glycosylation and sulfation (Hering and Sandell, manuscript submitted for publication) relate to this aspect of processing and assembly is not known. Many proteins and protein complexes require proper folding and oligomer formation for exit from the ER. Studies of two well-characterized viral glycoproteins, vesicular stomatitis virus G protein and influenza hemagglutinin, have documented in detail the relationship of folding and oligomerization to exit from the ER and have shown that a failure to achieve correct folding and oligomerization results in retention in the ER (Gething et al., 1986; Kreis and Lodish, 1986; Copeland et al., 1986, 1988; Doms et al., 1987). Similar relationships between conformational or oligomer

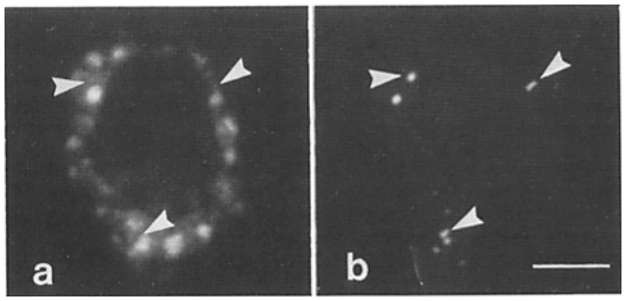

Figure 8. CSPG precursor-containing regions of the ER are distinct from the rER. The intracellular localization of antibodies against resident proteins of the rER (*a*) was compared with that of CSPG antibodies (*b*) in double immunofluorescence reactions. CSPG precursor-containing regions of the ER (*arrowheads*) are not reactive with the rER antibodies. Bar, 2 μm.

states and exit from the ER have been demonstrated for the T cell antigen receptor (Chen et al., 1988; Lippincott-Schwartz et al., 1988), immunoglobulins (Bole et al., 1986), and collagens (Koivu and Myllyla, 1987), among other membrane and secretory proteins. Both cotranslational and post-translational mechanisms are operative in the acquisition of native conformation and oligomer formation.

Finally, perhaps the ER subcompartment corresponds to a degradative, or predegradative region. In this case, the accumulated CSPG precursors may be collected or targeted for destruction because of some defect of folding or assembly. As discussed above, the retention and sometimes the degradation of incorrectly folded or assembled products and unassembled subunits in the ER have been documented for a number of multimeric complexes. Recent reports describing the nonlysosomal degradative processing of T cell antigen receptors in a compartment within or closely associated with the ER are most interesting in this regard (Chen et al., 1988; Lippincott-Schwartz et al., 1988).

In summary, we have described a subcompartment of the ER of cartilage cells that contains a relatively high concentration of CSPG precursors, and that apparently excludes type II procollagen. Our study presents clear evidence for the subcompartmentalization of the ER in untreated cells involved in the elaboration of their normal biosynthetic products. Current investigations are directed toward understanding the function of this interesting region of the chondrocyte ER.

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