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Xylosylation Is an Endoplasmic Reticulum to Golgi Event*

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The subcellular site of xylosylation, the first carbohydrate modification of the core protein that initiates glycosaminoglycan chain synthesis, was characterized *in situ*. Methods were developed to combine electron microscopic (EM) autoradiography and the radiolabeling of semi-intact chondrocytes. In the accompanying paper, Kearns *et al.* (Kearns, A. E., Vertel, B. M., and Schwartz, N. B. (1993) *J. Biol. Chem.* 268, 11097–11104) presented biochemical and subcellular fractionation studies that utilized semi-intact chondrocytes and radiolabeled UDP sugars to overcome obstacles to the direct analysis of xylosylation. The results suggested that xylosylation begins in the endoplasmic reticulum (ER) and continues in the Golgi. The site of xylosylation was not specified further due to the limitations of subcellular fractionation techniques. The studies described in this report were undertaken to localize these modifications directly *in situ*. Semi-intact cell preparations were optimized for ultrastructural preservation by modifications of permeabilization methods utilizing nitrocellulose filter overlays. Biochemical analysis demonstrated the exclusive incorporation of UDP-xylose into the cartilage chondroitin sulfate proteoglycan (aggrecan) core protein and 3′-phosphoadenosine 5′-phosphosulfate (PAPS) into the highly modified proteoglycan monomer. Immunolocalization studies showed the equivalence of cytoplasmic subcompartments in normal and semi-intact chondrocytes at the levels of light and electron microscopy. Once the biochemical and morphological equivalence of intact and semi-intact cells was established, EM autoradiographic studies were pursued using UDP-[^{3}H]xylose and [^{35}S]PAPS. Based on both qualitative and quantitative data, silver grains resulting from incorporated sulfate were concentrated in the perinuclear Golgi, while those resulting from incorporated xylose were found at the *cis* or forming face of the Golgi and in vesicular regions of the peripheral cytoplasm associated with the late ER. These data support the view that xylose addition begins in a late ER compartment and continues in intermediate compartments, perhaps including the *cis*-Golgi.

The production of normally functioning proteins often involves extensive co- and post-translational modifications catalyzed by specific enzymes within distinct subcellular compartments. Recent studies show that membrane and secretory proteins synthesized and processed by the endomembrane system may require carbohydrate addition, conformational changes, oligomerization, and perhaps other modifications in order to exit the ER[1] and progress through the secretory pathway (Rose and Doms, 1988; Hurtley and Helenius, 1989; Pelham, 1989; Farquhar, 1991).

Proteoglycans are useful models for the study of biosynthetic processing. Aggrecan, the large aggregating chondroitin sulfate proteoglycan of hyaline cartilage, has a core protein of greater than 250 kDa to which *N*-linked and *O*-linked oligosaccharides, and over 100 chondroitin sulfate and keratan sulfate glycosaminoglycan chains are attached covalently (reviewed by Wight *et al.* (1991), Hardingham and Fosang (1992)). It is clear that translation of the core protein and the addition of *N*-linked oligosaccharides are processes that occur in the RER, while the elongation and sulfation of glycosaminoglycan chains are functions of the *medial*- and *trans*-Golgi (Geetha-Habib *et al.*, 1984; Kimura *et al.*, 1984; Pacifici *et al.*, 1984; Vertel and Barkman, 1984; Ratcliffe *et al.*, 1985; Vertel *et al.*, 1985a, 1985b; Lohmander *et al.*, 1986; Vertel and Hitti, 1987; Campbell and Schwartz, 1988; Hascall *et al.*, 1991; Sugumaran and Silbert, 1991). In contrast, the sites for other processing reactions, such as the addition of linkage sugars that initiate glycosaminoglycan chain elongation, have not been as well characterized.

The *O*-linked addition of xylose to serine residues of the aggrecan core protein, catalyzed by the enzyme xylosyltransferase, is the reaction that initiates chondroitin sulfate chain formation. It has been proposed that xylose addition is important in the regulation of aggrecan biosynthesis (Schwartz *et al.*, 1974; Schwartz, 1976). As discussed by Kearns *et al.* (1993), much is known about the specificity and biochemical characteristics of xylosyltransferase and the xylosyltransferase reaction, but the subcellular site of xylose addition remains in question. Results of previous studies have in some cases favored the ER (Horwitz and Dorfman, 1968; Geetha-Habib *et al.*, 1984; Hoffmann *et al.*, 1984) and, in others, the Golgi (Kimura *et al.*, 1984; Lohmander *et al.*, 1986; Nuwayhid *et al.*, 1986; Lohmander *et al.*, 1989) as the site of xylosylation. Direct analysis has been precluded by the inability of UDP sugars to be transported across the plasma membrane.

Recent approaches using semi-intact cells as models have been successful in overcoming the permeability barrier of the plasma membrane and have permitted elegant *in vitro* anal-

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[1] The abbreviations used are: ER, endoplasmic reticulum; EM, electron microscopy; FITC, fluorescein isothiocyanate; HBSS, Hank's balanced salt solution; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; PBS, phosphate-buffered saline; RER, rough endoplasmic reticulum; SI, semi-intact; TRITC, tetramethyl rhodamine isothiocyanate.

yses of organellar structure and function and interorganelle transport (Beckers *et al.*, 1987; Balch, 1989; Beckers and Balch, 1989; Donaldson *et al.*, 1991; Schwaninger *et al.*, 1991; Robinson and Kreis, 1992). In the accompanying paper, Kearns *et al.* (1993) demonstrate that the semi-intact chondrocyte provides an excellent model for the study of xylosylation. Based on biochemical and subcellular fractionation studies, it was concluded that xylosylation begins in the ER and continues in the Golgi. A more specific characterization of the site of xylosylation was not feasible due to the limitations of these experimental techniques (*e.g.* the potential cross-contamination of membrane fractions and lack of Golgi membrane subfractionation).

The present studies were undertaken to localize the site of xylosylation directly. Electron microscopic (EM) autoradiography, which has proven to be a powerful tool for the determination of structure/function relationships in the cell, was incorporated in the analysis of radiolabeled semi-intact cells as a fruitful new approach to the investigation of this problem. Here we describe morphological and EM autoradiographic studies of xylosylation in semi-intact chondrocytes that extend the biochemical and subcellular fractionation studies of Kearns *et al.* (1993). Our results demonstrate that the addition of xylose to the aggrecan core protein begins in the ER and may continue in an early compartment of the Golgi.

EXPERIMENTAL PROCEDURES

Materials—Fertile White Leghorn chicken eggs were purchased from Sharp Sales (West Chicago, IL). Trypsin, Ham's F-12 medium, fetal calf serum, antibiotic-antimycotic mixture, and Hank's balanced salt solution (HBSS) were obtained from GIBCO-Bethesda Research Laboratories. En^{3}Hance, [^{35}S]methionine, UDP-[^{14}C]xylose, UDP-[^{3}H]xylose, UDP-[^{3}H]galactose, and [^{35}S]PAPS were products of Du Pont-New England Nuclear. HATF filters were obtained from Millipore Corp. 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 fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC) and goat anti-rabbit Fab coupled to peroxidase were obtained from Organon Teknika (Rockville, MD). Saponin, 1,4-diazobicyclo[2.2.2]octane, sodium borohydride, and diaminobenzidine were products of Sigma. Glutaraldehyde, osmium tetroxide, hydroxypropyl methacrylate, and Epon were purchased from Electron Microscopy Sciences (Fort Washington, PA). Ilford-4 emulsion was purchased from Polysciences (Warrington, PA).

Cell Culture—Cartilage cells were prepared from the sterna of 15-day-old chicken embryos (Cahn *et al.*, 1967) and grown directly on 60-mm gelatinized, tissue culture dishes or on carbon-coated coverslips at a density of 5×10^5 cells per dish in 3 ml of medium. For electron microscopy, cells were cultured onto gelatinized 35-mm tissue culture dishes at the same density.

Preparation of Semi-intact Cells—Chondrocytes grown in monolayer culture for 5–7 days were incubated for 10 min at 37 °C in testicular hyaluronidase to remove extracellular aggrecan (Vertel and Dorfman, 1979). (Initial experiments showed that proteoglycan in the cell-associated extracellular matrix prevented the necessary contact between the chondrocyte cell surface and nitrocellulose filter and consequently prevented the conversion of intact to semi-intact cells.) After hyaluronidase digestion, washes with phosphate-buffered saline (PBS), and the return to complete medium, cells were made semi-intact by a modification of published procedures (Donaldson *et al.*, 1991) using overlays of HATF filters presoaked in semi-intact incubation (SI) buffer (10 mM Hepes-KOH, pH 7.2, 125 mM potassium acetate, 2.5 mM magnesium acetate, 1 mM dithiothreitol, and 1 mg/ml glucose). The extent of drying prior to the application of the filter and time of incubation for the cell monolayer and nitrocellulose filter prior to peeling the filter free were adjusted for each experiment to optimize the number and quality of semi-intact cells. Cell permeabilization was evaluated by the uptake of trypan blue and observation in the phase microscope. Preparations containing greater than 90% semi-intact cells were used for further study.

Biosynthetic Labeling—Intact cells were washed twice with HBSS containing 10% Ham's F-12 medium at 37 °C and labeled at 37 °C with 50 μCi of [^{35}S]methionine in 200 μl or 25 μCi of $Na_2{}^{35}SO_4$ in 500 μl of the same medium. At the indicated times, aliquots were removed, centrifuged in a Microfuge, washed 3 times with cold HBSS, and treated as described below for immunoprecipitation or for gel electrophoresis. Semi-intact cells were washed with SI buffer several times before labeling in SI buffer containing isotope for 6 min at 37 °C. For biochemical experiments, semi-intact cells were labeled with 4 μCi of UDP-[^{3}H]xylose, 1 μCi of UDP-[^{14}C]xylose, 2 μCi of UDP-[^{3}H]galactose, or 4 μCi of [^{35}S]PAPS in 125 μl of SI buffer. Semi-intact cells used for EM autoradiographic analysis were labeled with 250 μCi of UDP-[^{3}H]xylose or 20 μCi of [^{35}S]PAPS in 100 μl of SI buffer. Aliquots (2 μl) of semi-intact cells used for autoradiographic studies were analyzed by gel electrophoresis (see below).

Immunoprecipitations—Intact and semi-intact cells were suspended in 2% SDS/HBSS, solubilized by boiling for 2 min, cooled quickly on ice, and diluted with 4 volumes of 50 mM Tris, pH 7.4, 190 mM NaCl, 6 mM EDTA, 2.5% Triton X-100. Indirect immunoprecipitations were performed by a modification (Vertel and Hitti, 1987) of the procedure described by Bumol and Reisfeld (1982). Briefly, antibodies were preadsorbed to Sepharose-Protein A. Immunoadsorbants were washed several times with immunoprecipitation buffer, incubated with labeled cell extracts overnight with shaking at 4 °C, washed extensively, and prepared for gel electrophoresis. This procedure resulted in the immunoprecipitation of >95% of the labeled aggrecan monomer and its precursors.

SDS/Polyacrylamide Gel Electrophoresis—Samples were solubilized in gel sample buffer (75 mM Tris-HCl, pH 6.7, 2% SDS, 20% glycerol, 0.003% bromphenol blue, 0.1 M dithiothreitol) by heating for 30 min at 70 °C and electrophoresed on 3–5% gradient polyacrylamide slab gels containing 0.1% SDS (Neville, 1971). Gels were treated with En^{3}Hance according to the recommendations of the manufacturer, dried, and processed for autoradiographic fluorography as described by Laskey and Mills (1975).

Immunofluorescent Staining—Semi-intact and intact cells were washed several times with SI buffer or PBS and fixed in 75% ethanol. Intact cells were incubated with testicular hyaluronidase for 10 min at 37 °C and washed with PBS several times to remove extracellular aggrecan prior to fixation as described (Vertel and Dorfman, 1979), in order to view intracellular, aggrecan-containing compartments. Fixed intact and semi-intact cells were rinsed with 75% ethanol, treated for 2 min with 98% ethanol/ether (1:1 v/v), and air-dried (von der Mark *et al.*, 1977) prior to immunofluorescence staining. Samples were incubated in primary antibodies for 40 min at room temperature, washed with HBSS, and incubated for 40 min with goat anti-rabbit, goat anti-rat, or goat anti-mouse IgG coupled to either FITC or TRITC. After further washes, samples were mounted in PBS/glycerol (1:9, v/v) containing 1,4-diazobicyclo[2.2.2]octane, observed using a Leitz Ortholux microscope with phase and epifluorescence optics, and photographed.

Antibodies—Polyclonal rabbit and guinea pig antibodies directed against hyaluronidase-digested aggrecan monomer were prepared and characterized previously (Upholt *et al.*, 1979; Vertel and Dorfman, 1979; Upholt *et al.*, 1981). The S103L rat monoclonal antibody recognizes an 11-amino acid epitope within the aggrecan core protein (Krueger *et al.*, 1990). These polyclonal and monoclonal antibodies recognize the cell-free translated aggrecan core protein and all intracellular and extracellular forms of aggrecan. Immunofluorescence staining properties of these antibodies and other characteristics of their immunoreactivities have been reported (Upholt *et al.*, 1979; Vertel and Dorfman, 1979; Upholt *et al.*, 1981; Vertel and Barkman, 1984; Vertel *et al.*, 1985a, 1985b; Vertel and Hitti, 1987; Vertel *et al.*, 1989). The monoclonal antibody 2-D-3, which recognizes keratan sulfate, was graciously provided by Dr. Bruce Caterson. The presence of this epitope within the perinuclear Golgi was demonstrated previously (Vertel and Barkman, 1984). Rabbit polyclonal antibodies against the 47-kDa heat shock protein were characterized (Saga *et al.*, 1987) and generously provided by Drs. Shinsuke Saga and Kenneth Yamada. These antibodies exhibit immunostaining of the ER.

Immunoperoxidase Staining and Electron Microscopy—The procedure described by Brown and Farquhar (1984) was used with some modifications. Intact or semi-intact chondrocytes were fixed in 0.15% glutaraldehyde/HBSS for 10 min at 25 °C, equilibrated with 0.1 M phosphate buffer, pH 7.4, and treated with 1% sodium borohydride for 30 min to quench unreacted aldehyde groups and restore antigenicity lost by glutaraldehyde fixation (Eldred *et al.*, 1983). Cells were permeabilized by incubation in 0.01% saponin/HBSS for 40 min and incubated in primary antibodies for 60 min at 37 °C in the presence of 0.01% saponin and 5% normal goat serum. Samples were washed

for 30 min with HBSS containing 0.01% saponin and incubated for 60 min at 37 °C in peroxidase-coupled goat anti-rabbit IgG Fab, diluted 1:75. Samples were washed for 40 min in saponin/HBSS prior to exchange with 0.1 M sodium phosphate, pH 7.4, 7.5% sucrose and incubation in 0.2% diaminobenzidine, 0.1 M sodium phosphate, pH 7.4, 7.5% sucrose for 5 min. Horseradish peroxidase-antibody products were visualized by a 3–15-min incubation in 0.2% diaminobenzidine, 0.1 M sodium phosphate, pH 7.4, 7.5% sucrose, containing 0.01% H_2O_2. After several washes in 0.1 M sodium cacodylate, pH 7.4, 7.5% sucrose, cells were fixed for 60 min in 2.5% glutaraldehyde, 0.1 M sodium cacodylate, pH 7.4, 7.5% sucrose, washed in 0.1 M sodium cacodylate, pH 7.4, 7.5% sucrose, postfixed at 4 °C for 45 min in 1% OsO_4, 1.5% $K_4Fe(CN)_6$, 0.1 M sodium cacodylate, pH 7.4, 7.5% sucrose, and treated at 25 °C for 30 min with 1% tannic acid, 0.1 M sodium cacodylate, pH 7.4, 7.5% sucrose prior to further processing for electron microscopy. Processing through embedment in Epon 812 followed the procedure of Brinkley *et al.* (1967). Sections were cut parallel to the plane of the cell monolayers on a Reichert ultramicrotome, usually counterstained with lead citrate, and observed in a Zeiss EM 10C transmission electron microscope.

Electron Microscopic Autoradiography—Semi-intact cells in monolayer were radiolabeled as described above, washed several times with SI buffer, and fixed for 1 h at room temperature with 2.5% glutaraldehyde in SI buffer. Several washes with SI buffer were followed by extensive washes with PBS conducted over 2 days. Samples were exchanged with 0.1 M cacodylate, pH 7.4, post-fixed for 1 h at 4°C in 1% OsO_4, 0.1 M cacodylate, pH 7.4, and treated for 30 min with 1% tannic acid in 0.1 M cacodylate, pH 7.4. Further processing for electron microscopy was according to the method of Brinkley *et al.* (1967), as described above. Sections cut parallel to the plane of the cell monolayers were collected on Formvar-coated, nickel grids, stained with uranyl acetate and lead citrate, and stabilized by carbon coating. Ilford 4 emulsion with a gold interference color was applied from a 1:3 dilution by a modified wire loop method described by Maunsbach (1966). Monodispersity of the coats was established by electron microscopy. Emulsion-coated grids were exposed at 4 °C in the dark for varying times (2–4 weeks for [^{35}S]sulfate and 6–9 months for [^{3}H]xylose) and developed for 2 min at 20 °C using Kodak D-19 developer.

For quantitative analysis, electron micrographs were photographed at a magnification of 5,000× and enlarged to a magnification of 20,000×. At least 75 cells and 1500 silver grains were counted for [^{35}S]sulfate-labeled semi-intact cells and for [^{3}H]xylose-labeled semi-intact cells. The location of each grain was determined from the smallest circle that could circumscribe it. Each silver grain was assigned to the following three subcellular compartments: perinuclear Golgi, non-Golgi cytoplasm, and nucleus. Relative distributions in the three compartments were compared for semi-intact cells labeled with [^{35}S]sulfate and those labeled with [^{3}H]xylose. The Student's *t*-test was used for statistical comparisons.

RESULTS

Optimization of Semi-intact Cell Preparations for Analysis by Autoradiographic Electron Microscopy—Xylose addition to the aggrecan core protein was demonstrated in the biochemical studies of semi-intact chondrocytes reported in the previous paper (Kearns *et al.*, 1993). That result led us to undertake complementary studies designed to characterize the subcellular site of xylosylation using electron microscopic autoradiography. In order to preserve optimal cell morphology, permeabilization was accomplished by the overlay of nitrocellulose filters in place of the hypotonic swelling and scraping method (Donaldson *et al.*, 1991). As shown in the phase micrograph of Fig. 1, chondrocytes remained attached to the substrate in characteristic isogenous groups. Even at the level of light microscopy, the nucleus and regions of the perinuclear Golgi are discerned (Fig. 1). Ultrastructural analysis reveals excellent structural preservation of the Golgi, ER, and other organelles (Fig. 2). For a morphological comparison with semi-intact cells prepared by the hypotonic swelling and scraping method, refer to Figs. 1 and 2 of Kearns *et al.* (1993).

Biochemical Properties of Semi-intact Cells—In order to determine whether nitrocellulose filter-permeabilized cells were able to incorporate UDP-xylose and PAPS into aggrecan, biochemical experiments comparable to those reported by Kearns *et al.* (1993) were performed. The *O*-linked addition of xylose to the aggrecan core protein in permeabilized cartilage cells using either UDP-[^{14}C]xylose or UDP-[^{3}H]xylose (Fig. 3*A*, *lanes 4* and *5*) yielded a product with an electrophoretic mobility identical with that of the core protein pulse-labeled with [^{35}S]methionine in intact chondrocytes (Fig. 3*A*, *lanes 1* and *3*). Sulfate from [^{35}S]PAPS was incorporated into a larger, more heterogeneous species (Fig. 3*A*, *lane 6*) which exhibited an electrophoretic mobility equivalent to that of the highly modified aggrecan monomer (Fig. 3*A*, *lane 2*). The identity of aggrecan precursors and intermediates in these experiments was established by immunoprecipitation with the monoclonal antibody, S103L (Fig. 3*A*, *lanes 3* and *4*), which recognizes all biosynthetic forms of aggrecan (Geetha-Habib *et al.*, 1984; Vertel and Hitti, 1987; Campbell and Schwartz, 1988; O'Donnell *et al.*, 1988). Direct analysis of biosynthetic products on higher percentage gels without immunoprecipitation showed that xylose was incorporated into the aggrecan core protein and no other proteins (Fig. 3*B*, *lane 2*). The exclusive incorporation of xylose into the aggrecan core protein established that *in situ* localization experiments utilizing this radioactive precursor would identify the intracellular sites of this biochemical event. Thus, EM autoradiography was pursued as a feasible method for the morphological identification of the subcellular sites of xylose and sulfate addition to aggrecan in chondrocytes. In contrast, UDP-[^{3}H]galactose was incorporated into the aggrecan core protein and several other glycoproteins, including type II procollagen (Fig. 3*A*, *lane 7*). Since UDP-[^{3}H]galactose labeled many glycoproteins, the specific incorporation into the aggrecan core protein could not be discriminated, and, therefore, studies of the intracellular site(s) of galactosylation were not continued.

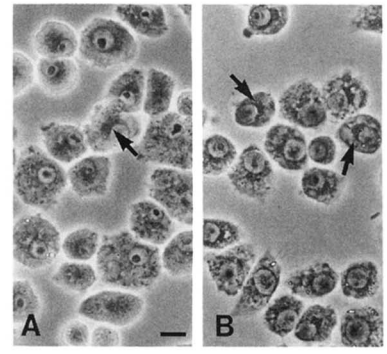

FIG. 1. **Intact and semi-intact chondrocytes are morphologically similar.** After 5 days of culture, semi-intact chondrocytes (*B*), prepared as described under "Experimental Procedures," were compared with intact chondrocytes (*A*). As shown, a similar morphology is exhibited in the phase contrast microscope. In both cases, groups of polygonal cells are observed. *Arrows* indicate the clearly defined perinuclear Golgi in intact (*A*) and semi-intact (*B*) cells. Calibration bar = 20 μm.

Equivalence of Subcellular Compartments and the Location of Antigens in Intact and Semi-intact Cells—The morphological and functional equivalence of subcellular compartments of intact and semi-intact cells was verified by immunolocalization studies, particularly with regard to aggrecan biosynthesis. Immunofluorescence staining of the perinuclear Golgi with 2-D-3, a monoclonal antibody that recognizes keratan sulfate chains known to be added to the aggrecan precursor in the Golgi, is observed for both intact (Fig. 4*A*) and semi-intact (Fig. 4*B*) cells. In contrast, antisera raised against the 47-kDa resident ER protein is shown to be localized in structures throughout the cytoplasm (presumably the ER) and excluded from the region of the perinuclear Golgi in intact

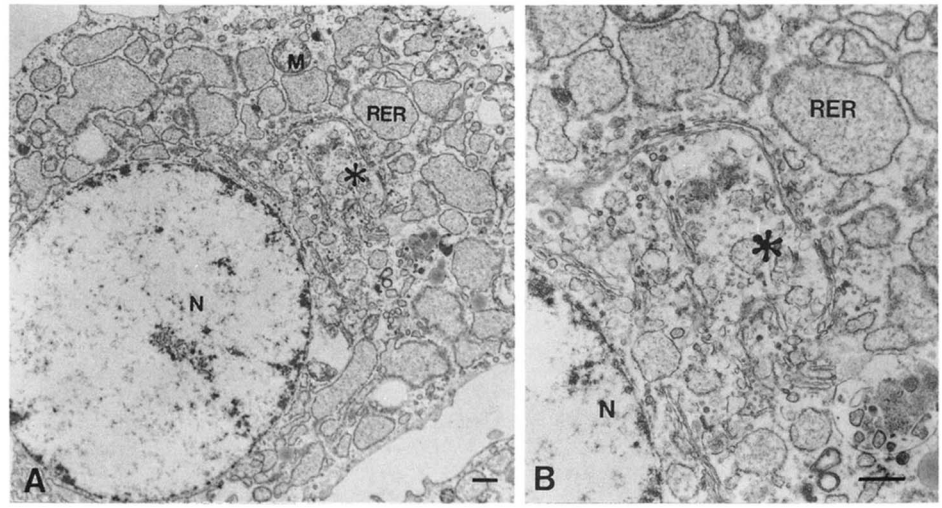

FIG. 2. **Ultrastructure is well-preserved in semi-intact chondrocytes.** When semi-intact chondrocytes prepared by the nitrocellulose filter overlay method (see "Experimental Procedures") are fixed directly in 2.5% glutaraldehyde, ultrastructural preservation is excellent. The Golgi region of the cell in *A* is shown at higher magnification in *B*. Morphological characteristics of the RER, Golgi (*asterisks*), nucleus (*N*), mitochondria (*M*), and other organelles are similar to those of intact chondrocytes. Spatial relationships are maintained among organelles and with the underlying substrate. Calibration bars = 0.05 μm.

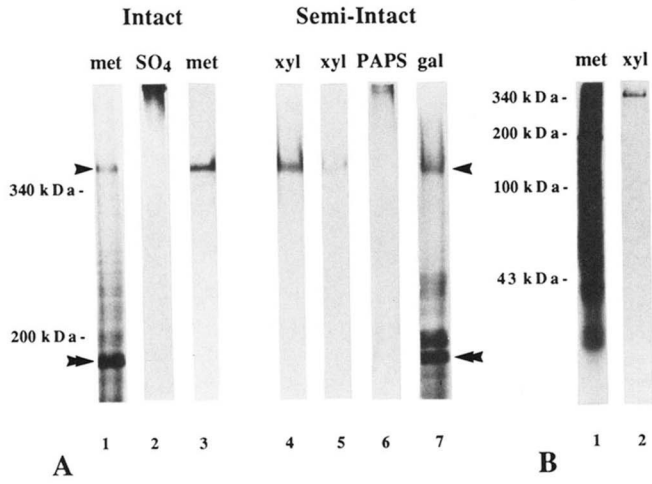

FIG. 3. **Characterization of aggrecan biosynthesis and modification in intact and semi-intact chondrocytes.** Intact (*A, lanes 1–3; B, lane 1*) and semi-intact (*A, lanes 4–7; B, lane 2*) chondrocytes were labeled with radioactive precursors, and products were displayed on SDS/polyacrylamide gradient gels composed of 3–5% (*A*) and 7.5–15% (*B*) polyacrylamide. Products of intact cells labeled with [^{35}S]-methionine (*A, lane 1* and *B, lane 1*) or [^{35}S]sulfate (*A, lane 2*) were prepared directly for SDS/PAGE and shown. The aggrecan core protein was immunoprecipitated with S103L from [^{35}S]methionine-labeled products and shown in *A, lane 3*. Products of semi-intact chondrocytes incubated with UDP-[^{14}C]xylose (*A, lane 4* and *B, lane 2*), UDP-[^{3}H]xylose (*A, lane 5*), [^{35}S]PAPS (*A, lane 6*), or UDP-[^{14}C]galactose (*A, lane 7*) followed by SDS-PAGE are shown. The xylosylated aggrecan core protein shown in *A, lane 4* was immunoprecipitated with S103L. Positions of the aggrecan core protein (*arrowheads*) and type II procollagen (*double arrowheads*) are indicated. Molecular masses (kDa), determined on the basis of protein standards, are on the *left*.

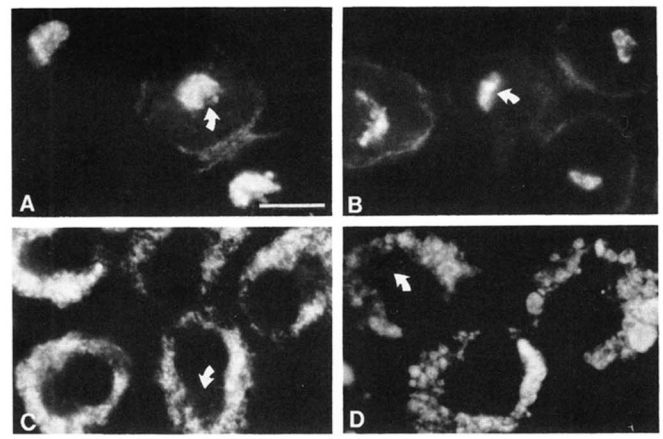

FIG. 4. **Equivalence of subcellular compartments in intact and semi-intact chondrocytes: light microscopic evidence.** Intact (*A* and *C*) and semi-intact (*B* and *D*) chondrocytes were used after 5 days of culture for immunofluorescence localization of the keratan sulfate (*A* and *B*) and the 47-kDa resident ER protein (*C* and *D*). The distribution of immunostained organelles is similar for intact and semi-intact cells; the 2-D-3 keratan sulfate monoclonal antibody reacts intensely with the perinuclear Golgi, while the ER polyclonal antibodies are localized throughout the cytoplasm, but absent from the region of the perinuclear Golgi. The region of the perinuclear Golgi is indicated by the *arrows*. Calibration bar = 10 μm.

(Fig. 4*C*) and semi-intact (Fig. 4*D*) cells. Ultrastructural analysis of semi-intact cells after immunoperoxidase reactions with the 2-D-3 anti-keratan sulfate (Fig. 5*A*) and anti-47-kDa protein (Fig. 5*B*) demonstrates reactivity of the Golgi and RER, respectively. Although immunolocalization within specific cytoplasmic compartments is unequivocal, we noted that ultrastructure was disrupted as a result of the modifications of fixation and permeabilization protocols required for immunostaining (compare ultrastructure of Figs. 2 and 5). Thus, in order to preserve optimal structure, considered crucial to the objectives of this study, the EM autoradiographic studies described below were performed on semi-intact cells fixed in 2.5% glutaraldehyde and not processed under conditions required for immunoelectron microscopic studies.

EM Autoradiography—Since sulfation is well established as a Golgi-mediated event in intact cells, we first used EM autoradiography to determine the subcellular site of sulfate incorporation in semi-intact cells. As shown in Fig. 6, grains resulting from the incorporation of [^{35}S]PAPS were concentrated in the region of the perinuclear Golgi. The quantitative analysis presented in Table I demonstrates that 83% of the silver grains were localized to the perinuclear Golgi, while 10% were observed in the non-Golgi cytoplasm. Grains located in the nucleus (7%) were considered to reflect nonspecific labeling. Thus, both qualitative and quantitative data identify the perinuclear Golgi as the site of sulfation in semi-intact cells, as is the case for intact cells.

For EM autoradiographic studies of xylosylation, UDP-[^{3}H]xylose at high specific activity was used to maximize incorporation and resolution. Grains were observed in peripheral regions of the cytoplasm as well as in the perinuclear Golgi (Fig. 7). In contrast to the sulfate incorporation data

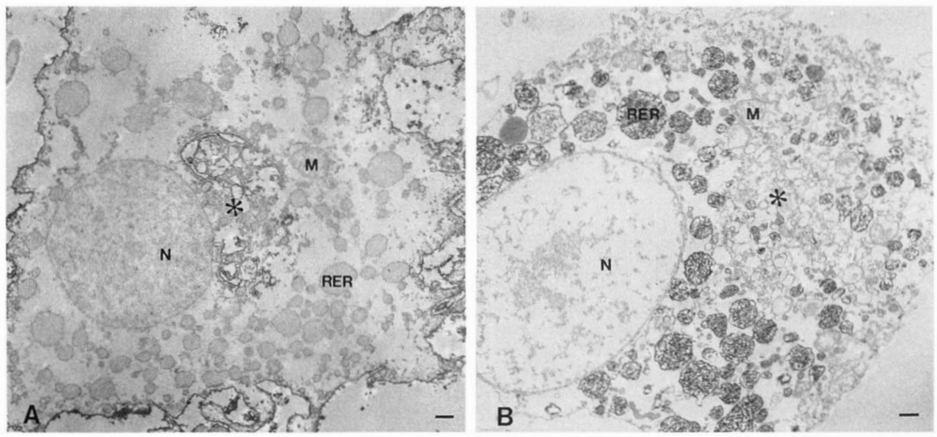

FIG. 5. **Immunoelectron microscopic characterization of cytoplasmic compartments of semi-intact chondrocytes.** Immunoperoxidase localization of keratan sulfate and the 47-kDa resident ER protein within semi-intact chondrocytes is shown in the electron micrographs of *A* and *B*, respectively. The perinuclear Golgi (*asterisks*) exhibits immunostaining for the 2-D-3 keratan sulfate monoclonal antibody, while antibodies to the 47-kDa protein react with lumenal contents of the RER in regions throughout the cytoplasm. The nucleus (*N*) and mitochondria (*M*), which exhibit no specific immunoreactivity, are also indicated. Note that the conditions for fixation required to retain reactive epitopes for immunoperoxidase localization compromise ultrastructural characteristics (compare structural features of Figs. 5 and 2). Calibration bar = 0.05 μm.

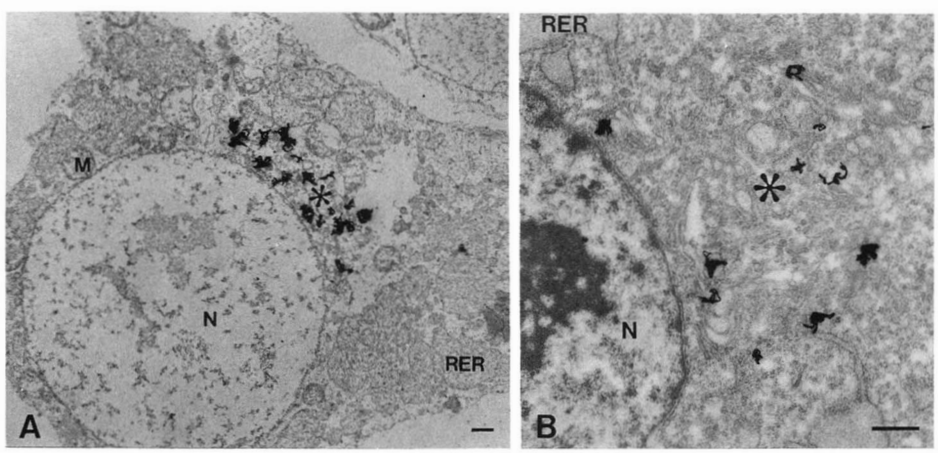

FIG. 6. **Semi-intact chondrocytes incorporate [^{35}S]PAPS in the perinuclear Golgi.** Silver grains that develop after incubation of semi-intact chondrocytes with [^{35}S]PAPS and processing for EM autoradiography are concentrated in the region of the perinuclear Golgi (*asterisks*). *A*, the low magnification electron micrograph demonstrates that grains are not present in the nucleus (*N*) or the non-Golgi cytoplasm. *B*, the association of grains with elements of the Golgi stacks is shown at higher magnification. The nucleus (*N*), mitochondria (*M*), and RER are indicated. Calibration bars = 0.05 μm.

TABLE I

Distribution of autoradiographic grains in subcompartments of semi-intact chondrocytes after labeling with [^{35}S]PAPS and UDP-[^{3}H]xylose

Values are means ± S.E. of percent grains within each designated subcompartment, generated from the analysis of 75 cells labeled with [^{35}S]PAPS and 80 cells labeled with UDP-[^{3}H]xylose. The statistical differences in percent grains occupying the Golgi and non-Golgi cytoplasm after labeling with [^{35}S]PAPS and UDP-[^{3}H]xylose have a level of significance of $p < 0.01$.

Cellular subcompartment	[^{35}S]PAPS	UDP-[^{3}H]xylose
	% grains	
Golgi	82.7 ± 0.6	44.9 ± 2.0
Non-Golgi cytoplasm	10.1 ± 0.6	48.8 ± 2.0
Nucleus	7.2 ± 0.8	6.4 ± 0.7

described above, only 45% of silver grains resulting from incorporated [^{3}H]xylose were observed in the perinuclear Golgi, and 49% were present in the non-Golgi cytoplasm (Table I). Labeling of the nucleus, indicative of nonspecific background, was low for xylose (6%) as well as for sulfate.

A more detailed examination of UDP-[^{3}H]xylose incorporation revealed several additional features about xylosylation compartments. As shown in Fig. 7*A*, some autoradiographic grains were observed in the region of the perinuclear Golgi. However, in contrast to the grains resulting from [^{35}S]PAPS incorporation, grains that developed from UDP-[^{3}H]xylose incorporation were often noted at the *cis*, or forming, face of the Golgi (Fig. 7*B*). Grains of the peripheral cytoplasm were associated with the ER, but many were located over vesicularizing structures at the periphery of the ER (*arrows*, Fig. 7). These observations suggest a late ER/early Golgi site for xylosylation. Interestingly, immunolocalization studies of intact chondrocytes have shown that aggrecan precursors are concentrated in smooth membrane-limited regions of the ER (Vertel *et al.*, 1989). Semi-intact cells are similarly characterized by the concentration of aggrecan precursors (*e.g.* the native xylosylation substrate) in restricted regions of the ER (Fig. 8).

DISCUSSION

Recent studies have highlighted the importance of ER-to-Golgi transitional regions for the regulation of anterograde, or forward movement through the secretory pathway, and retrograde, or reverse movement from the Golgi back to the ER (Pelham, 1989; Farquhar, 1991; Pelham, 1991; Klausner *et al.*, 1992; Rothman and Orci, 1992). Membrane recycling activities as well as biosynthetic functions have been ascribed to this zone, and several discrete intermediate compartments have been described (Saraste and Kuismanen, 1984; Rizzolo

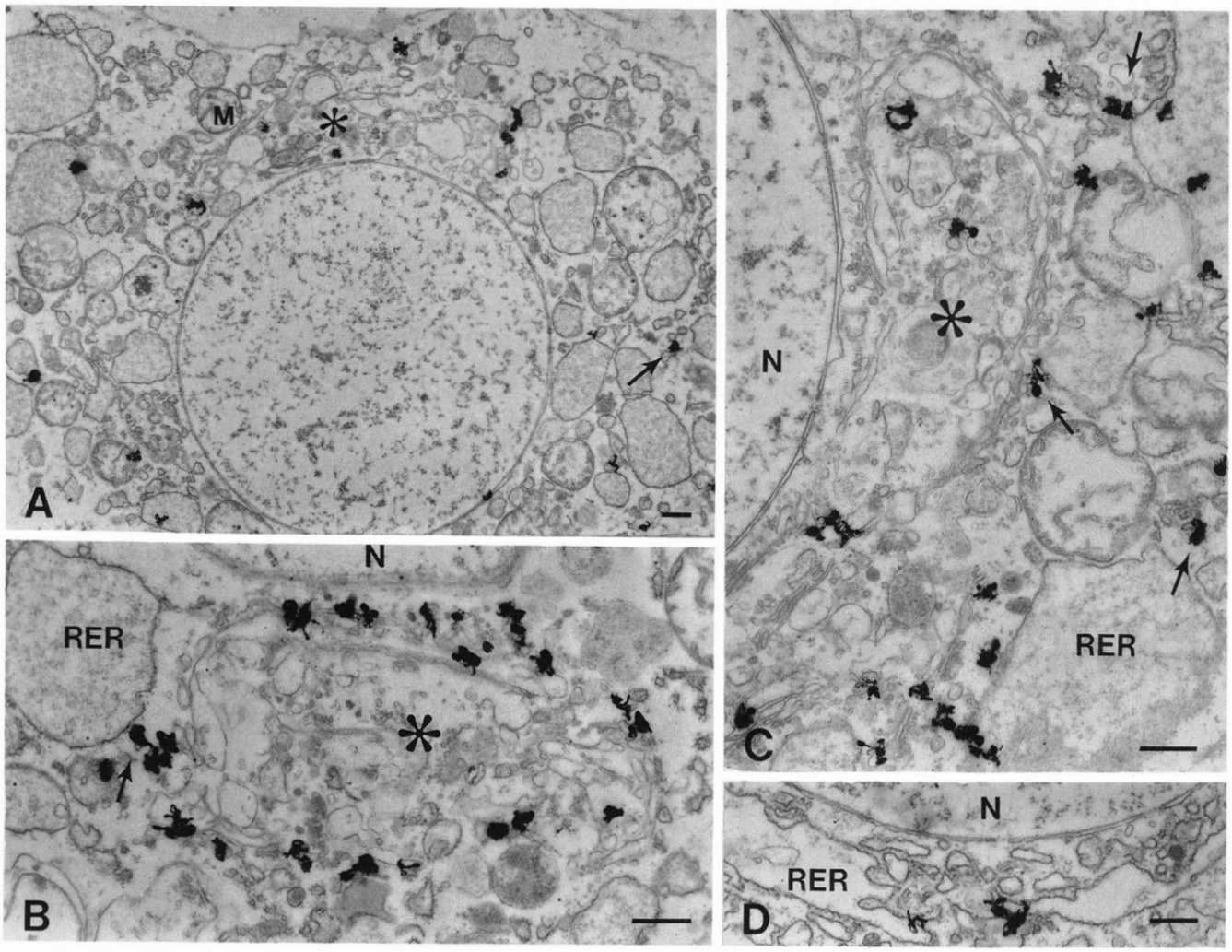

FIG. 7. **Semi-intact chondrocytes incorporate UDP-[^{3}H]xylose in peripheral ER regions and the perinuclear Golgi.** Silver grains that develop in semi-intact chondrocytes after autoradiographic localization of UDP-[^{3}H]xylose are observed in regions associated with the periphery of RER profiles (*arrows*) and the perinuclear Golgi (*asterisks*). *A*, at low magnification, grains are noted throughout the cytoplasm and in the perinuclear Golgi, but not in the nucleus (*N*). *B*, in this chondrocyte, silver grains encircle the Golgi, concentrated on the outer, or *cis*, face. Some grains are observed between the ER and Golgi (*arrow*). *C*, silver grains are frequently associated with vesicles at the periphery of the RER (*arrows*) and at the *cis*-Golgi. *D*, silver grains are located over vesicular edges of the RER in regions distant from the perinuclear Golgi as well. Calibration bars = 0.05 μm.

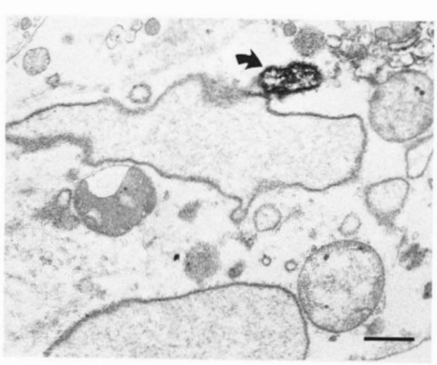

FIG. 8. **Aggrecan precursors are immunolocalized within peripheral regions of the ER in semi-intact chondrocytes.** The immunoperoxidase localization of antibodies to aggrecan and its precursors in semi-intact cells demonstrates reaction product in peripheral vesicular structures of the ER (*arrow*). Calibration bar = 0.05 μm.

et al., 1985; Warren, 1987; Tooze *et al.*, 1988; Vertel *et al.*, 1989; Schweitzer *et al.*, 1990; Saraste and Svensson, 1991; Schweitzer *et al.*, 1991; Hobman *et al.*, 1992; Huovila *et al.*, 1992; Kao and Draper, 1992; Lotti *et al.*, 1992; for review, see Farquhar (1991), Vertel *et al.* (1992)). Conflicting interpretations of subcellular fractionation studies directed our attention to the ER-to-Golgi interface as the potential site of xylosylation. Interestingly, in a previous immunohistochemical study of chicken chondrocytes, we observed the accumulation of aggrecan precursors in a late ER compartment (Vertel *et al.*, 1989). In the accompanying paper, Kearns *et al.* (1993) reported biochemical and subcellular fractionation studies of semi-intact chondrocytes and concluded that xylosylation begins post-translationally in the ER and continues in the Golgi where galactose, glucuronic acid, and sulfate are added. These provocative observations and results prompted us to pursue the *in situ* localization of xylosylation directly within the semi-intact chondrocyte using EM autoradiography.

Initial experiments were designed to establish the validity of this experimental approach. Light and electron microscopy

demonstrated the structural integrity of semi-intact cells and their organelles. The similar immunolocalization patterns exhibited by both semi-intact and intact chondrocytes for antibodies to the 47-kDa protein of the ER and keratan sulfate epitopes present in the Golgi indicated that the compartment-specific features of semi-intact and intact chondrocytes were comparable.

Further study was focused on the examination of incorporated UDP-[^{3}H]xylose and [^{35}S]PAPS after it was established that these precursors were exclusively transferred to the aggrecan core protein, while other precursors such as UDP-[^{3}H] galactose were utilized in the carbohydrate modifications of several glycoproteins. Using UDP-[^{3}H]xylose, we demonstrated that 49% of the observed grains were associated with the non-Golgi cytoplasm, while 45% were noted in the Golgi region. Often, the silver grains present in the Golgi region were closely associated with the *cis* face. The grains detected in the non-Golgi cytoplasm were frequently concentrated in peripheral, vesicularizing regions of the ER, suggestive of a late ER function. In contrast, 83% of the grains resulting from incorporated [^{35}S]PAPS exhibited localization within the region of the Golgi and appeared to be concentrated deeper in the stacks. Only 10% of the observed grains were associated with the non-Golgi cytoplasm. Thus, we conclude that the site of xylosylation differs significantly from the Golgi sulfation compartment and corresponds to the late ER/early Golgi, perhaps involving an intermediate compartment between the RER and *cis*-Golgi.

Other observations favor this interpretation as well. In the experiments presented, semi-intact cells were not provided with the cytosolic factors, energy-generating components, and other constituents required for interorganelle transport (Beckers *et al.*, 1987; Balch, 1989) and thus were not able to function in translocation. Since the semi-intact cells were not translocation-competent, it is likely that the site of incorporation (*e.g.* the late ER/early Golgi) either corresponds to the site of biosynthetic processing or is directly continuous with it. The relatively short period (<10 min) of labeling and low temperature washes prior to fixation also favors this interpretation. Finally, the comparative analysis of polysomes labeled with [^{35}S]methionine and UDP-[^{14}C]xylose reported by Kearns *et al.* (1993) lends support to the proposition that xylosylation is post-translational in the ER and continues in the early Golgi.

The apparent discrepancies among previous data can be resolved if we view xylosylation in chondrocytes as beginning in a late ER compartment and continuing in a pre- or early Golgi region. Presumably, the earlier subcellular fractionation studies suggesting xylose addition in the RER (Horwitz and Dorfman, 1968; Geetha-Habib *et al.*, 1984) utilized protocols that were not capable of distinguishing a unique subcompartment residing at the interface of the RER and Golgi and therefore led to the assignment of function predominantly in the RER or in the Golgi. Interestingly, Geetha-Habib *et al.* (1984) noted that core protein molecules modified by endoglycosidase H-sensitive, *N*-linked oligosaccharides functioned as xylose acceptors. This observation is compatible with xylosylation as a post-translational event, initiated in a late ER compartment. The immunoelectron microscopic localization of xylosyltransferase in the RER lumen of chicken chondrocytes supports the concept of RER-mediated xylosylation (Hoffmann *et al.*, 1984). However, the homogenization required to permeabilize paraformaldehyde-fixed cells for this particular immunolocalization technique could have led to the redistribution of enzyme within the ER compartment. In studies of Swarm rat chondrosarcoma cells using radioactive precursors and kinetic analyses, it was concluded that xylosylation is a Golgi-mediated process, perhaps occurring in an early Golgi compartment (Kimura *et al.*, 1984; Lohmander *et al.*, 1986, 1989). These data are also consistent with the post-translational, late ER/early Golgi addition of xylose. Although the data presented in this and the accompanying paper suggest that xylosyltransferase functions in a continuum of late ER/intermediate/early Golgi compartments, the level of activity within specific subregions may vary according to the physiological state of an individual cell or its particular phenotypic characteristics. In this regard, we observed some differences among individual chondrocytes in the relative distribution of autoradiographic grains for UDP-[^{3}H]xylose between the vesicular ER regions of the cytoplasm and the perinuclear Golgi (Table I).

Since proteoglycans represent several families of related macromolecules with unique functional properties and exhibit some variation in the kinetics and sequence of biosynthetic events, the possibility that different proteoglycan-synthesizing cells utilize different cytoplasmic subcompartments for the synthesis and processing of proteoglycans must also be considered. The cell-specific or phenotype-specific utilization of different subcompartments of the secretory pathway is suggested by studies of sialylation (Roth *et al.*, 1986; Roth, 1987; Lippincott-Schwartz *et al.*, 1989; Ulmer and Palade, 1989; Chege and Pfeffer, 1990; Sandvig *et al.*, 1991) and other enzyme activities. For example, evidence suggests that in the case of the *O*-linked addition of *N*-acetylgalactosamine, compartments between the RER and the *cis*-Golgi may be involved to different degrees. Several studies demonstrate that this type of *O*-glycosylation is initiated in the *cis*-Golgi (Roth, 1987; Deschuyteneer *et al.*, 1988; Perez-Vilar *et al.*, 1991). On the other hand, the modification was reported in a late ER/intermediate compartment for the E1 glycoprotein of the hepatitis A59 virus (Tooze *et al.*, 1988), in an ER subcompartment for mutant low density lipoprotein receptors (Pathak *et al.*, 1988), and in subcompartments of the RER in adenocarcinoma cells of the colon and in chicken chondrocytes (Perez-Vilar *et al.*, 1991). Thus, subdomains of the ER/intermediate compartment/early Golgi continuum may be involved differentially in the *O*-glycosylation required for the initiation of *O*-linked oligosaccharides.

Glycosaminoglycan chain synthesis commonly begins with the *O*-linked addition of xylose, a reaction that initiates the synthesis of chondroitin sulfate as well as heparan sulfate, and dermatan sulfate chains. Esko *et al.* (1985) presented genetic evidence that the same xylosyltransferase is used for heparan sulfate and chondroitin sulfate biosynthesis in Chinese hamster ovary cells, but the possibilities remain that different cell types utilize different subcellular compartments for xylosylation, and that different xylosyltransferase enzymes are involved in the xylosylation of different proteoglycans. The UDP-xylose transport/subcellular fractionation studies of Nuwayhid *et al.* (1986) suggest that in liver cells xylosylation occurs in the Golgi. Liver cells principally synthesize heparan sulfate proteoglycans, while chondrocytes are committed to the synthesis of chondroitin sulfate proteoglycans; thus, the sites of xylosylation in liver and cartilage cells may reflect differences in the nature of the proteoglycans synthesized. Alternatively, the demonstration of interconversion of nucleotide sugars within the ER lumen by Kearns *et al.* (1993) suggests that studies involving the transport of nucleotide sugars may have other explanations. Further investigation should reveal whether xylosylation occurs in the ER-to-Golgi intermediate compartments for all proteoglycan-synthesizing cells, or whether this enzymatic modification

characteristically occurs in other subcompartments in different types of proteoglycan-synthesizing cells.

In this report, we provide direct evidence of xylosylation in a late ER/intermediate/early Golgi compartment of chondrocytes by a novel combination of EM autoradiography and the use of semi-intact cells. These studies demonstrate that xylosylation occurs in that segment of the secretory pathway between the ER and Golgi in chicken chondrocytes, thereby suggesting a new function for this recently studied subcellular compartment. Current studies of intermediate compartments between the ER and Golgi are likely to reveal new insights about the structure, function, and organellar inter-relationships of this important segment of the secretory pathway.

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