Cell-free translation of messenger RNA for chondroitin sulphate proteoglycan core protein in rat cartilage

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Total RNA was extracted from the cartilage tissues rat Swarm chondrosarcoma, neonatal-rat breastplate and embryonic-chicken sterna and translated in wheat-germ cell-free reactions. The core protein of the chondroitin sulphate proteoglycan subunit was identified among translation products of rat mRNA by its apparent M_r of 330 000 and by its immunoprecipitation with specific antisera prepared against rat or chicken proteoglycan antigens. The apparent M_r of the rat proteoglycan core protein is 8000–10 000 less than that of the equivalent chicken cartilage core-protein product.

The extracellular matrix, which is characteristic of hyaline cartilage, contains abundant amounts of type II collagen (Miller, 1971) and chondroitin sulphate proteoglycan. Chondroitin sulphate proteoglycan monomers are associated with hyaluronic acid and link protein(s) in cartilage to form large macromolecular arrays (reviewed by Lindahl & Höök, 1978; Rodén, 1980; Dorfman, 1981; Hascall, 1981). Each proteoglycan monomer is composed of a protein core extensively modified by the addition of a variety of covalently bound carbohydrate side chains. Both *N*-linked and *O*-linked oligosaccharides have been demonstrated for chondroitin sulphate proteoglycan, in addition to the characteristic chondroitin sulphate and keratan sulphate side chains (Thonar & Sweet, 1979; De Luca *et al.*, 1980; Lohmander *et al.*, 1980). The proteoglycan monomer isolated from cartilage matrix has M_r 1×10^6 – 5×10^6 and contains 8–10% protein (Hascall & Riolo, 1972; Pasternack *et al.*, 1974). Although the enzymology of chondroitin sulphate side-chain synthesis has been studied extensively (Rodén, 1980), relatively little is known about the macromolecular intermediates formed during proteoglycan synthesis.

To examine directly the protein product of the core-protein gene in the absence of both proteolytic processing and the extensive carbohydrate

additions described above, we have translated RNA isolated from cartilage tissues in cell-free protein-synthesizing systems. We examined the translation products of chicken cartilage mRNA in previous studies (Upholt *et al.*, 1979, 1981). Because the Swarm rat chondrosarcoma has been used extensively in the study of chondroitin sulphate proteoglycan biosynthesis and proteoglycan-aggregate formation (Oegema *et al.*, 1975; Caterson & Baker, 1979; Kimura *et al.*, 1979, 1980, 1981; Fellini *et al.*, 1981; Kimata *et al.*, 1982), we chose to examine the translation products of rat cartilage mRNA too. In the present work we identify the putative core protein translated by using RNA prepared from the Swarm rat chondrosarcoma and neonatal-rat breastplate. The rat cartilage proteoglycan core protein translated from mRNA is compared with the corresponding chicken cell-free translation product, which has been described previously (Upholt *et al.*, 1979, 1981).

Materials and methods

Materials

Fertile White Leghorn hybrid chicken eggs were obtained from Sharpe Farms (Glen Ellyn, IL, U.S.A.). Wheat germ was kindly provided by General Mills (Vallejo, CA, U.S.A.). [35 S]-Methionine (580–1350 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, IL, U.S.A.). Collagenase (form III) was an Advance Biofactures (Lynbrook, NY, U.S.A.) product. Dynein was generously given by Dr. Frederick

Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; SDS, sodium dodecyl sulphate.

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Warner (Syracuse University). Antisera directed against rat chondrosarcoma chondroitin sulphate proteoglycan subunit digested with testicular hyaluronidase (EC 3.2.1.35), reduced and alkylated hyaluronidase-digested rat chondrosarcoma proteoglycan subunit and reduced and alkylated hyaluronidase-digested chicken cartilage proteoglycan subunit were generously given by Dr. Kazuyuki Sugahara (University of Chicago).

Preparation of RNA

Total RNA was prepared from the various cartilage tissues by modifications of the guanidium chloride extraction procedure (Cox, 1968) as described previously (Upholt *et al.*, 1979; Adams *et al.*, 1977). Tissues were removed, cleaned, quickly frozen in liquid N₂, stored at -70°C and later used for the isolation of RNA. Chicken sterna were dissected from 17-day embryos. Rat breastplates were dissected from Sprague-Dawley newborns 2 days after birth. Swarm rat chondrosarcomas were removed 3-4 weeks after tumour transfer. Tumours were maintained by intramuscular transplantation into Sprague-Dawley female rats (Stoolmiller *et al.*, 1975).

Cell-free translation

Wheat-germ cell-free translation reactions were performed as described previously (Upholt *et al.*, 1979). Proteins were precipitated with trichloroacetic acid for analysis by gel electrophoresis and for determination of protein synthesis by the method of Boedtker *et al.* (1976).

Immunoprecipitation and collagenase digestion of cell-free translation products

Antisera directed against several different chondroitin sulphate proteoglycan subunit preparations were used for the immunoprecipitation of cell-free translation products. Specific characteristics of the antibodies directed against hyaluronidase-digested chicken cartilage chondroitin sulphate proteoglycan subunit have been discussed elsewhere (Vertel & Dorfman, 1979). Properties of the antibodies directed against the hyaluronidase-digested rat chondrosarcoma proteoglycan subunit, and the reduction and alkylation of rat and chicken proteoglycan antigens, have been described by Sugahara & Dorfman (1979). Dorfman *et al.* (1980) have described the preparation and characterization of monoclonal antibodies specific for hyaluronidase-digested chicken cartilage chondroitin sulphate proteoglycan subunit.

Cell-free translocation products were diluted and centrifuged and the supernatants were immunoprecipitated by an indirect double-antibody precipitation method, as described previously (Upholt *et al.*, 1979).

Collagenase digestions of cell-free translated products were performed as described previously (Upholt *et al.*, 1979).

Polyacrylamide-gel electrophoresis

Samples were solubilized and reduced by heating at 100°C for 3 min in 0.1 mM-dithiothreitol/2% (w/v) SDS/80 mM-Tris/HCl (pH 6.8)/20% (v/v) glycerol. Electrophoresis was performed in 0.1% SDS with a discontinuous buffer system, a 3.1% (w/v) polyacrylamide stacking gel and a 5% (w/v) polyacrylamide resolving gel (Upholt *et al.*, 1979). Fluorographic analysis was performed by the procedure of Laskey & Mills (1975).

Results

RNA was extracted from Swarm rat chondrosarcoma and chicken sternal cartilage tissues and translated in wheat-germ extracts optimized for the synthesis of large polypeptides, including chicken cartilage proteoglycan core protein (Fig. 1). The cell-free translation product for chicken proteoglycan core protein has been previously identified (Upholt *et al.*, 1979, 1981) and migrates as a protein of M_r 340000, in a region of the gel devoid of other products. As shown in Fig. 1, the translation products of rat cartilage RNA (lanes 1 and 3) include a single polypeptide (labelled CP) in this M_r region of the gel, which migrates slightly ahead of the product directed by chicken mRNA (lanes 2 and 4). All of the other specifically indicated chicken and rat translation products in Fig. 1, except the rat band labelled a', are collagenase-sensitive (results not shown). When neonatal-rat breastplate RNA was translated, polypeptides equivalent to those directed by rat chondrosarcoma RNA were synthesized, suggesting that there are no major alterations in the sizes of the chondrosarcoma products as compared with those of the normal rat cartilage.

The core protein of the rat cartilage proteoglycan was identified by immunoprecipitation (Fig. 2) by using several different antisera directed against the following chicken or rat cartilage proteoglycan preparations: hyaluronidase-digested chicken cartilage chondroitin sulphate proteoglycan subunit (lane 1), hyaluronidase-digested rat chondrosarcoma proteoglycan subunit (lane 2), reduced and alkylated chicken proteoglycan (lane 4) and reduced and alkylated rat proteoglycan (lane 5). Each of these antisera can also precipitate the chicken core protein. An example of such an immunoprecipitated chicken core-protein translation product is shown for comparison in Fig. 2, lane 3. Neither the chicken nor the rat core protein is immunoprecipitated by normal rabbit serum or by antibodies directed against type II collagen

Fig. 1. Comparison of cell-free translation products directed by RNA from rat chondrosarcoma and chicken sternal cartilage

RNA was translated in wheat-germ cell-free reactions, and products were electrophoresed on 5%-polyacrylamide gels containing SDS as described in the Materials and methods section. Rat chondrosarcoma RNA was used at concentrations of 180 $\mu\text{g}/\text{ml}$ (lane 1) and 240 $\mu\text{g}/\text{ml}$ (lane 3), and chicken sternal cartilage RNA was used at concentrations of 160 $\mu\text{g}/\text{ml}$ (lanes 2 and 4). (a) Longer exposure of the top portion of the fluorographed gel shown in (b) displays both rat and chicken core proteins (CP). (b) Shorter exposure of the entire fluorographed gel displays more clearly the bands in the 100,000–200,000- M_r range. Collagenase-sensitive bands (b'–e' and a–e) and one additional rat product (a') are indicated. The M_r scale is shown on the left.

Fig. 2. Immunoprecipitation of core proteins synthesized by cell-free translation

Products directed by rat chondrosarcoma RNA (160 $\mu\text{g}/\text{ml}$) were immunoprecipitated as described in the Materials and methods section by using antibodies prepared against hyaluronidase-digested chicken cartilage chondroitin sulphate proteoglycan subunit (lane 1), hyaluronidase-digested rat chondrosarcoma proteoglycan subunit (lane 2), reduced and alkylated chicken proteoglycan (lane 4) and reduced and alkylated rat proteoglycan (lane 5). Cell-free synthesized chicken cartilage core protein is shown immunoprecipitated by antibodies directed against hyaluronidase-digested cartilage proteoglycan subunit in lane 3 for comparison.

(results not shown). A monoclonal antibody, $\bar{S}103L$ (Dorfman *et al.*, 1980), which immunoprecipitates the chicken cell-free translation core-protein product (Upholt *et al.*, 1981), does not precipitate the rat core-protein precursor even if 100 times the amount of antibody effective in the immunoprecipitation of the chicken product is used (results not shown; see the Discussion section).

The rat core protein reproducibly migrates at a slightly faster rate through SDS/polyacrylamide gels than does the chicken core protein (Figs. 1 and 2). These two products were electrophoresed in alternate lanes to demonstrate this difference clearly. When rat and chicken translation products were co-electrophoresed in the same lane, two distinct core protein bands could be resolved (results not shown). The slightly faster migration rate for the rat core protein suggests that the rat protein has M_r approx. 8000–10,000 less than that of the chicken core protein. Apparent M_r values were calculated by using a standard curve of electrophoretic mobility versus M_r for several proteins of known M_r , as described previously (Upholt *et al.*, 1979). The dynein standard (M_r 330,000) consistently migrates slightly ahead of the chicken

core protein. Determinations of apparent M_r were based on the assumption that electrophoretic mobility truly reflects the M_r of a protein. However, this assumption must be viewed with caution, as some proteins, notably the collagens (Freytag *et al.*, 1979; Sandell & Veis, 1980), behave anomalously on SDS/polyacrylamide gels, and it is possible that the core proteins might also migrate anomalously. The apparent M_r of the cell-free translated core protein was determined to be 330 000 for the rat and 340 000 for the chicken.

Discussion

The core protein of rat cartilage chondroitin sulphate proteoglycan subunit was identified among products translated from neonatal-rat breastplate and Swarm-chondrosarcoma mRNA by its specific immunoreactivity with different antisera directed against rat or chicken cartilage proteoglycan antigens. The M_r of 330 000 for the rat core protein was similar to, but not identical with, the M_r of 340 000 for the previously identified (Upholt *et al.*, 1979, 1981) core protein of chicken cartilage proteoglycan.

The clearest proof of identity for the core protein of the chicken proteoglycan was provided by its specific immunoprecipitation with one monoclonal antibody, S103L, which also immunoprecipitates [35 S]sulphate-labelled hyaluronidase-digested chicken cartilage proteoglycan (Upholt *et al.*, 1981). The presumptive rat core protein is not immunoprecipitated by the S103L monoclonal antibody. Rat cartilage proteoglycan does not inhibit the immunoprecipitation by S103L of chicken proteoglycan in inhibition assays (K. Sugahara & A. Dorfman, unpublished work), suggesting that S103L reactivity is species-specific. However, the demonstration of specific reactivity for both the rat and chicken core proteins with each of a variety of polyclonal antibodies directed against different proteoglycan antigens strongly suggests their similarity.

Kimura *et al.* (1981) have reported a M_r of 370 000 for the intracellular core-protein intermediate synthesized by rat chondrosarcoma cells in culture. They detected no change in the electrophoretic mobility of this intracellular intermediate with various times of chase after a pulse of radio-labelled amino acid. It is probable that this proteoglycan core-protein intermediate, in contrast with the unmodified cell-free translation product, has had a signal polypeptide removed (Blobel, 1980) and contains added *N*-linked and possible *O*-linked oligosaccharides (Lohmander *et al.*, 1980) in various stages of completion. These added carbohydrate groups could account for the apparent 40 000-Da increase in size of the intracellular core-

protein intermediate as compared with the cell-free translation product described in the present paper. However, the exact numerical value of this difference cannot be determined, since these molecules have not been compared directly in the same gel system. By the direct comparison of electrophoretic mobilities on the same calibrated gel, we have determined that the intracellular core-protein intermediate synthesized by chicken sternal chondrocytes in culture has an apparent M_r approx. 15 000 larger than that of the chicken cartilage mRNA-directed cell-free translation product (Vertel, 1983; B. M. Vertel & W. B. Upholt, unpublished work).

The cell-free core-protein products described here and related biosynthetic intermediates synthesized by cultured chondrocytes (Kimura *et al.*, 1981) appear to be of uniform size, in contrast with the considerable heterogeneity exhibited by matrix-associated proteoglycans isolated from cartilage tissues. Our results are consistent with the studies by Fellini *et al.* (1981) and Kimura *et al.* (1981), which suggest that the heterogeneity of rat chondrosarcoma proteoglycan molecules reflects the heterogeneity of attached chondroitin sulphate chains and not heterogeneity of the protein portion of the molecule.

In conclusion, we have identified the cell-free translation product corresponding to rat cartilage proteoglycan core protein and have shown that it has an M_r approx. 10 000 less than that of the equivalent chicken product.

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