Monoclonal Antibodies as Probes for Determining the Microheterogeneity of the Link Proteins of Cartilage Proteoglycan*

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Monoclonal antibodies were raised against Swarm rat chondrosarcoma link protein 2. Two of the resultant hybridomas (9/30/6-A-1 and 9/30/8-A-4) were used in structural analyses of the link proteins. The 9/30/6-A-1 monoclonal antibody recognized an epitope which was only present on rat chondrosarcoma link protein 2. This epitope was absent in rat chondrosarcoma link protein 3 obtained after trypsin or clostridial digestion of rat chondrosarcoma proteoglycan aggregate, indicating that proteolytic digestion either removed or modified the epitope. Contrastingly, the 9/30/8-A-4 monoclonal antibody recognized an epitope present in link protein(s) 1, 2, or 3 isolated from cartilage of several animal species (rat, bovine, human, and chicken). Rat chondrosarcoma link protein 2 was digested with Staphylococcus aureus V8 protease, and the resulting peptides were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subjected to immunolocation analyses. The 9/30/6-A-1 and 9/30/8-A-4 monoclonal antibodies recognized epitopes in two different halves of the link protein molecule. The 9/30/8-A-4 monoclonal antibody was used to identify proteolytic cleavage peptides common to the individual link proteins (1, 2, or 3) purified from cartilage proteoglycans of several animal species. Digestion of rat chondrosarcoma link protein 2 with endoglycosidase H or α-mannosidase increased its electrophoretic mobility to that of link protein 3 and removed or altered the determinant recognized by the 9/30/6-A-1 monoclonal antibody, indicating that a high-mannose oligosaccharide chain was part of the antigenic determinant. The 9/30/8-A-4 monoclonal recognition of epitope was unaffected by endo- or exoglycosidase treatment. Endo- and exoglycosidase treatment of bovine nasal cartilage link proteins also altered their electrophoretic mobility, indicating that high-mannose oligosaccharide structures on the various link proteins (1, 2, or 3) accounted for the microheterogeneity observed in sodium dodecyl sulfate-polyacrylamide gels.

Proteoglycans of hyaline cartilages occur as large macro-molecular aggregate structures composed of proteoglycan monomers, hyaluronic acid, and link protein(s) (1–4). The specificity of the interaction of these three components, their biochemical characterization, and the methods for their extraction and isolation have been recently reviewed (5, 6). The occurrence of the “link proteins” in bovine nasal cartilage proteoglycan aggregate was first demonstrated by Keiser et al. (7), and their structural dissimilarity from proteoglycan monomer has been demonstrated in both biochemical (8) and immunological analyses (9–11).

Within the proteoglycan aggregate structure, the link proteins serve the function of stabilizing the association of the proteoglycan monomers with the central core of hyaluronic acid (12, 13). The link proteins can specifically associate with both the proteoglycan monomers (14) and the hyaluronic acid (14, 15). The presence of the link proteins in the proteoglycan aggregate results in an increased resistance of the complex to thermal denaturation and enzymatic degradation (12, 13).

Three link proteins have been identified in bovine nasal cartilage proteoglycan aggregate: link protein 1, link protein 2, and a minor component link protein 3, with molecular weights of 49,000, 45,000, and 43,000, respectively (16). Similarly, three link proteins with molecular weights ranging from 43,000 to 50,000 have been isolated from human articular cartilage (17, 18). Only one link protein (link protein 2) occurs in proteoglycan aggregate isolated from Swarm rat chondrosarcoma (19). The structural similarity of the link proteins from bovine nasal cartilage has been demonstrated by peptide map analysis (16, 20, 21) and in immunological studies (22). The major difference in their biochemical analyses resides in their relative carbohydrate content (16), and these differences are thought to, in part, account for their apparent heterogeneity after SDS-polyacrylamide gel electrophoresis (23). Studies by Roughley et al. (17) have indicated that sialic acid residues on the link protein(s) account for some of the polydispersity and microheterogeneity of the link proteins isolated from human articular cartilage.

Little is known of the primary structure of the link proteins. Pépin et al. (24) have reported amino acid sequence data for the first 20 amino acids of the N-terminal of the bovine nasal cartilage link protein 3 which results from trypsin or clostridial digestion of bovine nasal cartilage proteoglycan aggregate (3, 25). Sequence analyses of link protein 3 resulting from clostridial digestion of rat chondrosarcoma proteoglycan aggregate has been recently reported (26) and indicate that there is considerable homology with the amino acid sequence of bovine nasal cartilage link protein 3 (24).

Polyclonal antibodies have been used in immunological analyses of link protein for quantitation (9–11, 27), for im-

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† The abbreviations used are: SDS, sodium dodecyl sulfate; SDS-PAGE, polyacrylamide gel electrophoresis in SDS; A1, A1D1, and A1D4, proteoglycan aggregate proteoglycan monomer, and link protein-rich fraction, respectively, from equilibrium density gradient fractionation procedures for purifying proteoglycan substructures as described by Heinegard (36).
Monoclonal Antibodies to Link Proteins

Materials—All reagents used were of analytical reagent grade. *S. aureus* V8 protease, endoglycosidase H, endoglycosidase D, neuraminidase, N-acetylglucosaminidase, and β-galactosidase were obtained from Miles Laboratories. α-Mannosidase was purchased from Boehringer Mannheim. Bovine serum albumin and Coomassie Brilliant Blue R-250 was obtained from Sigma and Nonidet P-40 from Gallard-Schlesinger. Fluorescamine (Fluram) and dry acetone (manuf- age) were obtained from Fisher. Nitrocellulose paper (Grade BA 85) used for electrophoretic transfer ("electroblotting") of proteins was obtained from Schleicher & Schuell and [125I]Hodine was bought from Amersham Corp.

**Polyacrylamide Gel Electrophoresis—SDS-PAGE** was performed in 10% (10 x 0.9) and 15% (15 x 0.9) polyacrylamide gels in the presence of 0.1% SDS using the procedure described by Laemmli (34). Ten per cent gels (1.5-mm thick containing 10 sample wells and a 3-cm-long 3.2% stacker gel) were used for fractionation of the link protein(s) in AID4 fractions. Fifteen per cent gels (1.5-mm thick containing 10 sample wells with a 4.5-cm long 3.2% stacker gel) were used for fractionation of peptides resulting from *S. aureus* V8 protease digestion of the link protein(s). Fluorescamine labeling of samples was used to locate bands resolved by SDS-PAGE (16). Fluorescamine in dry acetone (20 μl of a freshly prepared 0.1% solution) was added rapidly with vigorous mixing to the sample dissolved in an aqueous solution of 2% SDS in 50 mM sodium bicarbonate, pH 8.1.

**Isolation of Cartilage Proteoglycans and Link Protein(s)—** Proteoglycan aggregate (A1), monomer (A1D1), and a fraction enriched in the link protein(s) (A1D4) were extracted and isolated from hyaline cartilages obtained from different animal species and tissues, by methods described in previous publications (16, 35, 37). Swarren chondrosarcoma, bovine nasal cartilage, chick sternal cartilage, and human articular cartilage were the sources of proteoglycan and link protein preparations used in the experiments described in this manuscript.

**Purified rat chondrosarcoma link protein (link protein 2)** was obtained after chromatography of the AID4 fraction (20 mg) on a column (100 x 1 cm diameter) of Sepharose CL-6B eluted with 4 M guanidine HCl, 0.05 M sodium acetate, pH 6.8. Column fractions were monitored by absorbance at 290 nm. The elution profile was similar to that obtained using 1% SDS as the eluting buffer (16, 35) and achieved a separation of link proteins from protein-rich proteoglycan material in the AID4 fraction similar to that described by Tang et al. (10). The presence of link protein in appropriate fractions was demonstrated by SDS-PAGE analyses of small aliquots of each of the column fractions (16). Fractions containing purine prior to rat chondrosarcoma link protein were pooled, concentrated to approximately 1 mg/ml by vacuum dialysis, and then dialyzed against 0.15 M NaCl for 24 h at 4°C. Aliquots of this link protein suspension were used to immunize BALB/c mice as described below.

**Immunization, Fusion, and Cloning Procedures—** The protocol for immunization, fusion, and cloning has been described in recent publications (37, 38). Initial identification of positive clones recognizing rat chondrosarcoma link protein 2 was obtained by enzyme immunoassay analyses of hybridoma media. A similar method of enzyme immunoassay analysis was used to further determine the specificity of the monoclonal antibodies to their immunoglobulin isotype (37). Hybridoma cells producing monoclonal antibodies directed against rat chondrosarcoma link protein were injected interleptically into female BALB/c mice (retired breeders), and ascites fluid was obtained for large scale purification of the monoclonal immunoglobulin.

**Characterization of the Specificity of the Monoclonal Antibodies to Link Protein**—The specificities of the 9/30/6-A-1 and 9/30/8-A-4 monoclonal antibodies were determined using a modification of the Western blot procedure described by Burnette (39). AID4 fractions obtained from rat chondrosarcoma, bovine nasal cartilage, human articular cartilage, and chick sternal cartilage were subjected to SDS-PAGE in 10% gels using the electrophoresis system described by Laemmli (34). Each series of the four AID4 fractions was run in triplicate. After SDS-PAGE separation of the proteins in the AID4 samples, one-third of the gel containing a set of the fractionated link proteins was cut and stained with Coomassie Blue to identify all of the proteins present in each AID4 preparation. The remaining gel was used for electrophoretic transfer of duplicate series of the AID4 fractions to nitrocellulose. Conditions used in the electrophoretic transfer of proteins to nitrocellulose were as described by Burnette (39). After transfer, the nitrocellulose strip was "blocked" by incubating for 1 h at 4°C with 1.5% bovine serum albumin, Tris-saline buffer. The incubation with the 125I-labeled monoclonal ascites fluid was performed in 150-mm disposable plastic Petri dishes (50 ml/dish) with gentle rotary shaking (60 rpm) for 1 h at room temperature. The nitrocellulose sheets were washed sequentially in Tris-saline buffer (10 min), 0.05% Nonidet P-40, Tris-saline buffer (20 min and then air-dried between Whatman 3MM Whatman paper. Finally, the Whatman paper strips were wrapped in Saran Wrap and exposed to x-ray film (Kodak X-Omat AR) for 6-24 h at ~70°C. Localization of the electrophoblot proteins recognized by each 125I-labeled immunoglobulin was achieved after this autoradiography procedure. Identification of specific proteins was achieved by comparing the autoradiographs with the Coomassie-stained gel.

*S. aureus* V8 Protease Digestion of Link Protein(s) and Binding of Specific Peptides to the 9/30/6-A-1 and 9/30/8-A-4 Monoclonal Antibodies—The method used for the digestion of link protein(s) with *S. aureus* V8 protease and localization of the resultant peptides is a modification of the procedure described in an earlier publication (16). V8 protease was adapted from the protocol described by Maitz et al. (40), i.e. an AID4 sample (20 μg) dissolved in 2% SDS, 50 mM sodium bicarbonate (20 μl) was labeled with fluoroscamine (16). The fluoroscamine-labeled AID4 was mixed with untreated AID4 (80 μg) and the fractionated by SDS-PAGE on a 10% gel. After electrophoresis the proteins of the AID4 fraction were visualized by exposure to long wavelength ultraviolet light, and portions of the gel containing link protein bands 1, 2 or 3 were excised from the gel and placed in the sample wells of another gel (15% polyacrylamide) which had been furnished with an extra-long stacker gel (~4.5 cm). *S. aureus* V8 protease mixtures (50 μl) containing different amounts of enzyme (no enzyme, 0.1, 0.5, and 2.5 μg of enzyme, respectively. The electrophoresis sample buffer) were layered over each gel piece. Electrophoresis was at 5 mA/gel while the sample traversed the stacker gel (1-2 h) and thereafter 15 mA/gel. Replicates of each series (no enzyme, 0.1, 0.5, and 2.5 μg of enzyme, respectively) were made in order to allow for replicate analyses of Coomassie staining of the peptide pattern and analyses by Western blotting and immunolocalization with the 125I-labeled 9/30/5/A-1 and 9/30/8-A-4 monoclonal antibodies as described in the previous section.

**Treatment of Link Proteins with Endo- and Exoglycosidases—** Link protein preparations (AID4) were succinylated (41) to render them water-soluble prior to their treatment with endo- or exoglycosidases. AID4 (5 mg) was suspended in 1.5 ml of 1 M NaCO3, pH 8.0. Succinic anhydride (3 x 2 mg) was added while the suspension was stirred at room temperature. The solubilization of link protein was continued for 1 h at room temperature and then the reaction mixture was dialyzed against phosphate-buffered saline-azide (0.15 M NaCl, 0.02%...
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pendent upon the relative amount of antigen that binds to compared. In this analysis, positive controls using monoclonal antibodies recognizing epitopes on proteoglycan substructures respectively. Both of these antibodies bound clostripain treatment of rat chondrosarcoma proteoglycan. The 913016-A-1 immunoglobulin did not recognize any determinants present on the epitope recognized by different monoclonal antibodies. In indicated that the immunoglobulins produced by the 913016-A-1 and 913018-A-4 hybridomas were performed in triplicate. One set of replicates was stained with Coomassie Blue; the other two were electroblotted to nitrocellulose and antigenic proteins were identified by immunolocalization using the 9/30/6-A-1 and 9/30/8-A-4 monoclonal antibodies, respectively.

RESULTS AND DISCUSSION

Determination of Monoclonal Antibody Specificity—Rat chondrosarcoma link protein 2 was used as the antigen for the production of the 9/30/6-A-1 and 9/30/8-A-4 hybridomas. Enzyme immunoassay analyses for antibody isotype (37) indicated that the immunoglobulins produced by the 9/30/6-A-1 and 9/30/8-A-4 hybridomas were IgG, and IgGp, respectively. Both of these antibodies bound S. aureus protein A and thus could be used in radioimmunoassay procedures utilizing heat- and formalin-inactivated S. aureus (9, 33, 42, 43).

Enzyme immunoassay analyses of the specificity of the 9/30/6-A-1 and 9/30/8-A-4 immunoglobulins were performed (Fig. 1). Antigens coated to the enzyme immunoassay plate were obtained from rat chondrosarcoma, bovine nasal cartilage, and chick sternum cartilage. Enzyme immunoassay analyses were performed as described in Ref. 37. Undiluted hybridoma supernatants were used as the source of the 9/30/6-A-1 and 9/30/8-A-4 monoclonal antibodies. The relative intensity of antibody recognition of antigen(s) was determined by measurement of absorbance at 405 nm. The solid bar indicates antigen recognized by the 9/30/6-A-1 monoclonal antibody. The open bars indicate antigens recognized by the 9/30/8-A-4 monoclonal antibody. Antigens: RC, rat chondrosarcoma; BNC, bovine nasal cartilage; ChS, chick sternum; L1, L2, and/or L3, link proteins 1, 2, and 3, respectively; HABR, hyaluronic acid binding region isolated from cartilage proteoglycans by procedures described in Ref. 25; HABR/LP3, the ternary complex of hyaluronic binding region, hyaluronic acid, and link protein 3 (25); A1, link protein-stabilized proteoglycan aggregate (30); core (ABC), proteoglycan core protein produced after chondroitinase ABC digestion of proteoglycans (43).

The specificities of the 9/30/6-A-1 and 9/30/8-A-4 monoclonal immunoglobulins were further defined after SDS-polyacrylamide gel electrophoretic separation of the link proteins, electrophoretic transfer to nitrocellulose, and immunolocalization as described under “Experimental Procedures.” The electrophoretic mobility of the link proteins (link pro-
Monoclonal Antibodies to Link Protein(s)

a. SDS-PAGE

b. 6-A-1

c. 8-A-4

FIG. 2. Determination of monoclonal antibody specificity by electrophoretic transfer to nitrocellulose and immunolocation analyses. Link protein-containing fractions (A1D4) from rat chondrosarcoma (RC), bovine nasal cartilage (BNC), human articular cartilage (HAC), and chick sternal cartilage (ChS) were subjected to SDS-PAGE fractionation. These separations were performed in triplicate. a, SDS-PAGE gel stained with Coomassie Blue. The notations 1, 2, and 3 denote the migration of link protein(s) 1, 2, or 3 from the various tissue sources. b, replicate SDS-PAGE fractionation of A1D4 sample, where the separated proteins have been transferred to nitrocellulose and subjected to immunolocation analyses using 125I-labeled 9/30/6-A-1 monoclonal antibody (see “Experimental Procedures”). c, replicate SDS-PAGE and immunolocation analyses using 125I-labeled 9/30/8-A-4 monoclonal antibody.

teins 1, 2, and/or 3) from rat chondrosarcoma, bovine nasal cartilage, human articular cartilage, and chick sternal cartilage are indicated on the Coomassie-stained gel (Fig. 2a). Replicate gels were run, the separated proteins were transferred to nitrocellulose, and proteins that were recognized by the 125I-labeled 9/30/6-A-1 and 9/30/8-A-4 monoclonal antibodies were identified by the immunolocation procedure of Burnette (39). The results are shown in Fig. 2b and indicate that the 9/30/6-A-1 monoclonal antibody specifically recognizes a determinant present on rat chondrosarcoma link protein 2 that is not present on link protein(s) isolated from other animal species. The slower migrating band also recognized by the 9/30/6-A-1 antibody is an oligomeric form of link protein which appears after prolonged storage of link protein in SDS solutions.2 Contrasting this result, the 9/30/8-A-4 monoclonal antibody recognizes a determinant present on link protein(s) 1, 2, or 3 from a variety of animal species (Fig. 2c). This result indicates that the determinant recognized by the 9/30/8-A-4 monoclonal antibody may be part of a conserved sequence of the link protein polypeptide common to all link proteins and represents an important structural or functional domain of the link protein primary sequence. Both the 9/30/6-A-1 and the 9/30/8-A-4 monoclonal antibodies recognized epitopes on link protein(s) fractionated on SDS-PAGE gels run in the presence of 10% β-mercaptoethanol (results not shown). The results above (Fig. 2) confirm the specificities of the monoclonal antibodies that were determined by enzyme immunoassay analyses (Fig. 1).

The link protein peptides containing the antigenic determinants recognized by the 9/30/6-A-1 and 9/30/8-A-4 monoclonal antibodies were identified after digestion of rat chondrosarcoma link protein 2 with S. aureus V8 protease, separation by SDS-polyacrylamide gel electrophoresis, electrophoretic transfer to nitrocellulose, and immunolocation (Fig. 3). Coomassie staining of a resultant SDS-polyacrylamide gel indicated the presence of 5 major peptides as products of the S. aureus V8 protease digestion of rat chondrosarcoma link protein 2 (Fig. 3a). The peptides (denoted a–e) have apparent molecular masses of 25, 20, 17, 10, and 7 kDa, respectively. Replicate SDS-polyacrylamide gels were run and subjected to

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2 An oligomerization of link protein(s) occurs with prolonged storage in SDS. The occurrence of this minor component is apparent in the autoradiogram using the 9/30/6-A-1 monoclonal antibody for immunolocalization (Fig. 2b). Peptide map analyses on 125I-labeled A1D4 from the same preparation have been performed to show that this band is indeed oligomeric link protein (B. Caterson, unpublished observation). This phenomenon did not occur with freshly prepared A1D4 fractions and immunolocation analyses (see Fig. 6).
Monoclonal Antibodies to Link Protein(s)

Fig. 3. Identification of S. aureus V8 protease peptides of rat chondrosarcoma link protein 2 that contain the antigenic determinant recognized by the 9/30/6-A-1 and 9/30/8-A-4 monoclonal antibodies. Peptides containing epitopes recognized by each antibody were identified by overlaying the exposed autoradiograms on the dried Coomassie-stained gel. a, Coomassie Blue-stained SDS-PAGE gel showing the peptides resulting from digestion of rat chondrosarcoma link protein with no enzyme, and 0.1, 0.5, and 2.5 \( \mu g \) of S. aureus V8 protease, respectively. Five peptides (a–e) are indicated. E shows the location of the S. aureus V8 protease. b, immunolocation using \(^{125}\)I-labeled 9/30/6-A-1 monoclonal antibody. Peptides a and c contain the epitope recognized by this monoclonal antibody. c, immunolocation using \(^{125}\)I-labeled 9/30/8-A-4 monoclonal antibody. Peptides b, d, and e contain the epitope recognized by the 9/30/8-A-4 monoclonal antibody.

The placement of the antigenic determinant, recognized by either 9/30/6-A-1 or 9/30/8-A-4, on the rat chondrosarcoma link protein 2 and that the epitope pattern of whether peptide 4 monoclonal antibody. The placement of the antigenic determinant recognized by the 9/30/6-A-1 monoclonal antibody. The peptide containing this epitope must be removed or modified in the clostripain digestion of link protein in aggregate (Fig. 4).

Additional analyses were made of the peptides containing the 9/30/8-A-4 determinant after S. aureus V8 protease digestion of rat chondrosarcoma link protein 2, bovine nasal cartilage link protein(s), and chick sternal cartilage link protein(s) (Fig. 5). Separately isolated link proteins (1 or 2) from rat chondrosarcoma, bovine nasal cartilage, and chick sternal cartilage proteoglycans were digested with 2.5 \( \mu g \) of S. aureus V8 protease, and the peptides were separated by electrophoresis, electrophoretically transferred to nitrocellulose, and identified by immunolocation using the \(^{125}\)I-labeled 9/30/8-A-4 monoclonal antibody. The resultant peptide pattern is shown in the Coomassie-stained gel (Fig. 5a). Several major peptides are evident in the S. aureus V8 protease digests of link proteins from each animal species. The peptide pattern for rat chondrosarcoma link protein 2 is similar to that shown in Fig. 3a for digestion with 2.5 \( \mu g \) of S. aureus V8 protease and thus can be used for comparison of the peptide patterns from the bovine and chick link proteins. The similarity in the peptide patterns for link proteins 1 and 2 from bovine nasal cartilage or chick sternal cartilage indicates structural similarities in the link proteins from each individual animal species. However, the peptide pattern for the link proteins of bovine nasal cartilage is noticeably different from that of the rat chondrosarcoma or chick sternal link proteins. Immunolocation with \(^{125}\)I-labeled 9/30/8-A-4 monoclonal antibody is shown in Fig. 5b. The immunolocation pattern for the V8 protease peptides of rat chondrosarcoma link protein 2 is similar to that seen in Fig. 3c. However, an additional minor peptide (Mr 19,000) is resolved more clearly in this gel separation. This intermediate peptide is more easily recognizable in the peptide maps of link protein(s) from bovine and chicken cartilage proteoglycans. The 9/30/8-A-4 monoclonal antibody recognizes four peptides common to the link proteins of different animal species and also to each link protein subpopulation from a given animal species. The results indicate that

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peptides recognized by the 913018-A-4 monoclonal antibody (Fig. 1). Peptides generates the smaller link protein determinants on the two peptides (25 and peptide maps of chicken sternal link proteins epitope recognized by the 913018-A-4 monoclonal antibody.

Clostripain digestion of rat chondrosarcoma proteoglycan aggregate there is considerable similarity in the chicken sternal link proteins (25 and 17 kDa, respectively) contain the antigenic determinant recognized by the 9/30/6-A-1 monoclonal antibody whereas peptides b, d, and e (20, 10, and 7 kDa, respectively) contain the epitope recognized by the 9/30/8-A-4 monoclonal antibody.

there is considerable similarity in the S. aureus V8 protease peptide maps of chicken sternal link proteins 1 and 2 and that of rat chondrosarcoma link protein 2. The peptide patterns from bovine nasal cartilage link proteins 1 and 2 have electrophoretic mobilities different from those for the chick and rat chondrosarcoma link protein peptides. More recent studies have shown similarities in the S. aureus V8 protease peptide map patterns of link proteins (1, 2, and 3) from human articular cartilage and bovine nasal cartilage. These results indicate the presence of common cleavage sites (aspartic and/or glutamic acid) in the amino acid sequence of rat and chicken link proteins that are different from those present in human and bovine link proteins.

Endo- and Exoglycosidase Treatment of Link Protein(s) and Their Resultant Immunoreactivity with the 9/30/6-A-1 and 9/

FIG. 4. Model for identification of peptides resulting from proteolytic digestion of rat chondrosarcoma link protein 2 (RC-L2) with S. aureus V8 protease and clostripain. RC-L2 is shown with the two antigenic determinants recognized by the 9/30/6-A-1 and 9/30/8-A-4 monoclonal antibodies. The placement of the determinants on the two peptides (25 and 20 kDa) is arbitrary. Clostripain digestion of rat chondrosarcoma proteoglycan aggregate generates the smaller link protein (RC-L3, M, 43,000). Enzyme immunoassay analysis indicates that RC-L3 only contains the epitope recognized by the 9/30/8-A-4 monoclonal antibody (Fig. 1). Peptides a and c (25 and 17 kDa, respectively) contain the antigenic determinant recognized by the 9/30/6-A-1 monoclonal antibody whereas peptides b, d, and e (20, 10, and 7 kDa, respectively) contain the epitope recognized by the 9/30/8-A-4 monoclonal antibody.

30/8-A-4 Monoclonal Antibodies—Recent studies using polyclonal antibodies directed against bovine nasal cartilage link proteins have indicated that the antigenic determinants recognized by the polyclonal rabbit immunoglobulins were in regions of the molecule where carbohydrate moieties (N- and O-link oligosaccharides) were attached to the link protein polypeptide (26). Experiments were designed to examine whether any of the monoclonal antibodies generated against rat chondrosarcoma link protein 2 were recognizing determinants associated with the carbohydrate oligosaccharides of the link protein(s). Swarn rat chondrosarcoma link protein 2 (M, 45,000) was digested with endoglycosidase H, α-mannosidase, or a series of exoglycosidases (neuraminidase, β-galactosidase, N-acetylhexosaminidase) followed by endoglycosidase D. For these experiments a rat chondrosarcoma A1D4 preparation was succinylated with succinic anhydride (41) to render the link protein water-soluble, facilitating the removal of the carbohydrate chains from the glycoprotein by the endo-
FIG. 6. Digestion of succinylated rat chondrosarcoma link protein 2 with endoglycosidases and/or exoglycosidases followed by SDS-PAGE fractionation of the treated samples. Replicates of lanes 1 through 5 show (a) the Coomassie Blue-stained gel, (b) immunolocation analyses with the 125I-labeled 9/30/6-A-1 monoclonal antibody, or (c) the 125I-labeled 9/30/8-A-4 monoclonal antibody. Lane 1, no enzyme treatment; lane 2, digestion with neuraminidase; lane 3, digestion with neuraminidase, β-galactosidase, N-acetylhexosaminidase, and endoglycosidase D; lane 4, digestion with endoglycosidase H; lane 5, digestion with α-mannosidase. The arrows indicate the electrophoretic mobility of rat chondrosarcoma link protein 2 and 3 on SDS-PAGE.

and exoglycosidases. The results are shown in Fig. 6. Analyses of the Coomassie-stained SDS-PAGE gel indicate that treatment with neuraminidase, β-galactosidase, N-acetylhexosaminidase, and endoglycosidase D has no effect on the electrophoretic mobility of rat chondrosarcoma link protein. However, treatment of rat chondrosarcoma link protein 2 with endoglycosidase H or α-mannosidase results in an increase in the mobility of the link protein 2 to that of the faster moving link protein 3. This result indicates that rat chondrosarcoma link protein 2 contains a high-mannose oligosaccharide chain, the removal of which increases the mobility of the protein to a position similar to that of link protein 3 produced by clostrpin or trypsin treatment of rat chondrosarcoma proteoglycan aggregate (25).

Replicate gels were run, electrophoretically transferred to nitrocellulose, and subjected to immunolocation analyses using 125I-labeled 9/30/6-A-1 or 125I-labeled 9/30/8-A-4 monoclonal antibody. The results are shown in Fig. 6, b and c, respectively. Treatment with neuraminidase, β-galactosidase, N-acetylhexosaminidase, and endoglycosidase D (lanes 2 and 3, Fig. 6b) did not alter the antigenicity of rat chondrosarcoma link protein 2 to the 9/30/6-A-1 immunoglobulin. However, digestion of rat chondrosarcoma link protein 2 with endoglycosidase H (lane 4, Fig. 6b) removes or modifies the antigenic determinant recognized by the 9/30/6-A-1 immunoglobulin. Similarly, digestion of rat chondrosarcoma link protein 2 with α-mannosidase also results in an increase in the mobility of rat chondrosarcoma link protein 2 to that of rat chondrosarcoma link protein 3 and removal of the antigenic determinant recognized by 9/30/6-A-1 (lane 5, Fig. 6b). The immunoreactive material remaining after endoglycosidase H or α-mannosidase digestion (Fig. 6b, lanes 4 and 5) represents small amounts of undigested link protein 2. Contrasting this, the specificity of the 9/30/8-A-4 immunoglobulin is unaffected by digestion of carbohydrate chains of rat chondrosarcoma link protein 2 with any of the endo- or exoglycosidases. Link protein 3, produced by digestion of link protein 2 with either endoglycosidase H or α-mannosidase, contained the epitope recognized by the 9/30/8-A-4 monoclonal antibody (lanes 4 and 5, Fig. 6c). These results indicate that the 9/30/6-A-1 is recognizing a high-mannose oligosaccharide chain possibly in association with the polypeptide backbone of rat chondrosarcoma link protein 2. It is unlikely that the antibody is just recognizing the carbohydrate moiety, as high-mannose oligosaccharide structures are common to many glycoproteins. The above studies indicate that the presence or absence of high-mannose chains on rat chondrosarcoma link protein influences its relative mobility in SDS-PAGE gels.

Additional experiments were performed to examine whether endo- and exoglycosidase treatment of the link proteins from bovine nasal cartilage also influenced their electrophoretic mobility on SDS-PAGE gels. Bovine nasal cartilage link protein(s) (1, 2, and 3) were succinylated and then treated with the various glycosidases prior to immunolocation with 125I-labeled 9/30/8-A-4 monoclonal antibody. The results are shown in Fig. 7. These results show that incomplete or partial removal of oligosaccharides from the different subpopulations of link proteins alters their electrophoretic mobility. Examination of the Coomassie-stained gel (Fig. 7a) indicates that treatment with either endo- or exoglycosidases causes a redistribution of the proportion of link proteins 1, 2, or 3 in the SDS-PAGE gel separations. Immunolocation using the 9/30/
8-A-4 immunoglobulin (Fig. 7b) clarifies this point as the link protein banding pattern is unaffected by other proteins present in each SDS-PAGE separation (the commercial preparations of the endo- and exoglycosidases contain bovine serum albumin and other proteins to maintain the stability of the enzymes). Densitometric scans were performed on both the Coomassie-stained gel and the autoradiogram resulting from immunolocation with [125I]-labeled 9/30/8-A-4 antibody given in Fig. 7. These results are shown in Fig. 8 and are expressed as the relative proportion of link proteins 1, 2, and 3 present after each given enzyme treatment. The analyses indicate that treatment of bovine nasal cartilage link protein 1, 2, or 3 with neuraminidase, β-galactosidase, N-acetylhexosaminidase, and endoglycosidase D cause only small changes in their mobility in SDS-polyacrylamide gel electrophoresis. There appears to be a minor conversion of link protein 1 to link protein 3; link protein 2 seems less affected by this treatment. (Compare lane B with lane A in Figs. 7 and 8.) The major changes in electrophoretic mobility of the link proteins occur when they are treated with either endoglycosidase H or α-mannosidase. (Compare lanes C and D with lane A in Figs. 7 and 8.) Both bovine nasal cartilage link protein 1 and bovine nasal cartilage link protein 2 are susceptible to digestion with these enzymes, resulting in an increased proportion of link protein with the electrophoretic mobility similar to that of link protein 3. These results indicate that high-mannose oligosaccharide structures on the protein polypeptide of link proteins 1 and 2 account for the microheterogeneity that is observed in the link proteins of proteoglycan aggregate isolated from bovine nasal cartilage (i.e. the presence of 1, 2, and/or 3 link protein(s)). The biological significance of these different link protein subpopulations in hyaline cartilage preparations needs to be further investigated.

**GENERAL DISCUSSION**

The data presented in this paper describe the characterization of the specificity of two monoclonal antibodies directed against the primary structural components of rat chondrosarcoma link protein 2. One of the monoclonal antibodies, 9/30/6-A-1, recognizes a determinant present only on rat chondrosarcoma link protein 2. This antigen appears to be specific for rat chondrosarcoma link protein 2 as it did not recognize determinants in link protein preparations isolated from rat articular cartilage (results not shown).

Treatment of rat chondrosarcoma link protein 2 with endoglycosidase H or α-mannosidase results in the removal of a high-mannose oligosaccharide chain and alters the electrophoretic mobility of the link protein 2 to one that is similar to link protein obtained after clostripain treatment of proteoglycan aggregate (link protein 3). Removal of the high-mannose oligosaccharide also removes or modifies the antigenic determinant recognized by the 9/30/6-A-1 monoclonal antibody. Similarly, enzyme immunoassay analyses (Fig. 1) and immunolocation analyses (results not shown) indicate that the 9/30/6-A-1 monoclonal antibody does not recognize an antigenic determinant present on clostripain-generated link protein 3. These results indicate that the antigenic determinant (epitope) recognized by the 9/30/6-A-1 monoclonal antibody consists of a high-mannose oligosaccharide on the link protein polypeptide and that during clostripain treatment of proteoglycan aggregate a small peptide containing this oligosaccharide is removed from the link protein (see Fig. 4). Removal of this peptide (that presumably contains a high-mannose oligosaccharide) increases the electrophoretic mobility of the resultant link protein 3.

The second antibody, 9/30/8-A-4, recognized a determinant present on link protein(s) (1, 2, or 3) isolated from cartilage of a variety of animal species (rat, human, bovine, and chicken cartilage), suggesting that it was recognizing a determinant present in conserved regions of the link protein structure. Analyses of the immunoreactivity of the 9/30/6-A-1 and 9/30/8-A-4 monoclonal antibodies with peptides resulting from *S. aureus* V8 protease digestion of link protein indicated that they were recognizing determinants in different domains of the link protein polypeptide. Immunolocation analyses using these two monoclonal antibodies facilitated the identification of precursor peptides in the sequential degradation of the link protein(s) with *S. aureus* V8 protease.

Digestion of rat chondrosarcoma with endo- or exoglycosidases did not affect the epitope recognized by the 9/30/8-A-4 monoclonal antibody. The 9/30/8-A-4 monoclonal antibody was used to immunolocate bovine nasal cartilage link protein(s) that had been treated with endo- and exoglycosidases. These studies indicated that the presence of high-mannose chains on bovine nasal cartilage link proteins primarily accounted for the microheterogeneity that is observed in the fractionation of hyaline cartilage link protein(s) by SDS-polyacrylamide gel electrophoresis. Tunicamycin treatment of rat chondrosarcoma cells grown in tissue culture results in the synthesis of a link protein with an electrophoretic mobility similar to that of link protein 3 (44). This link protein is presumably devoid of oligosaccharides (complex and high-mannose) on the polypeptide, further indicating that the presence of such structures account for a decreased electrophoretic mobility of the resultant link protein 3.

**Fig. 8.** Densitometric scans of the Coomassie Blue-stained gel (open bars) and the autoradiogram from the [125I]-labeled 9/30/8-A-4 monoclonal antibody immunolocation analyses (solid bars) shown in Fig. 7. Analyses are presented as the relative proportion of link proteins 1, 2, and 3 present after (A) no enzyme treatment, (B) digestion with neuraminidase, β-galactosidase, N-acetylhexosaminidase and endoglycosidase D, (C) digestion with endoglycosidase H, or (D) digestion with α-mannosidase.
phoretic mobility in SDS-PAGE gels. These results indicate that there may be two forms of link protein 3 with identical electrophoretic mobilities: one derived from proteolytic cleavage (25) and the other devoid of carbohydrate (44). Removal of complex oligosaccharides from the link proteins showed minor changes in the redistribution of the link proteins in SDS-polyacrylamide gels. Studies by Roughley et al. (17) indicate that the removal of sialic acid residues on complex oligosaccharides on link proteins 1, 2, and 3 isolated from human articular cartilage simplifies the multiple banding patterns (and thus apparent microheterogeneity) of link protein in isoelectric focusing gels. However, this treatment did not significantly change the relative proportion of link proteins 1, 2, and 3 similar to our observations with bovine nasal cartilage link proteins. Recent studies on the effect of chemical deglycosylation of human articular cartilage link proteins have indicated that their microheterogeneity is markedly simplified by removal of carbohydrate.

As suggested in earlier studies (16) the microheterogeneity of link proteins (1, 2, and 3) from different cartilage tissues is primarily due to the presence of oligosaccharide structures on a common polypeptide. The studies described in this paper more clearly establish this previous conclusion and indicate that it is the presence of high-mannose oligosaccharide structures on the link proteins that account for the microheterogeneity that is observed after SDS-polyacrylamide gel electrophoresis. The reason for different link protein subpopulations within a given tissue is not understood. However, their presence may be related to the different proteoglycan subpopulations that have been shown to occur in cartilaginous tissues (46, 47).²

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² J. S. Mort, B. Caterson, A. R. Poole, and P. J. Roughly, manuscript in preparation.
³ Antibodies described in this manuscript will be made available to scientific investigators upon request.

Monoclonal Antibodies to Link Protein(s)

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