

INVARIANT CHAIN IS THE CORE PROTEIN OF THE Ia-ASSOCIATED CHONDROITIN SULFATE PROTEOGLYCAN

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The class II antigens of the major histocompatibility complex (MHC)¹ are critical to the generation of an immune response. They control the magnitude of the immune response of antigens and direct interactions among immunocompetent cells. Despite their well-documented role in a variety of immune phenomena, the biochemical mechanisms of Ia-mediated immune effects is not clearly understood at this time. Biochemical analyses indicate that Ia antigens consist of a 33–35 kD α chain glycoprotein and a 25–27 kD β chain glycoprotein, both of which are encoded by genes within the MHC. Over the past several years, additional components have been found to be noncovalently associated with the Ia heterodimer. The most extensively characterized of these is a 31 kD glycoprotein, termed invariant chain (Ii), which specifically associates with cytoplasmic forms of murine Ia antigens (1). Molecules homologous to invariant chain have since been identified in association with the human, rat, and guinea pig class II antigens (2–5). Although studied extensively by a number of laboratories, the function of the invariant chain in the biology of Ia has eluded investigators.

In our studies of the biochemistry of MHC class II antigens, we have identified an additional molecule associated with the Ia heterodimer (6–9). Biochemical studies demonstrated this molecule to be a chondroitin sulfate proteoglycan (CSPG) that has an apparent molecular weight of 46,000–70,000 (46–70 K) when analyzed by SDS-PAGE. This CSPG has been isolated from both murine and human lymphoid tissue and appears to be uniquely associated with class II antigens and not other lymphoid cell molecules. The class II-associated CSPG is considerably smaller than proteoglycans that have been isolated from other

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¹ *Abbreviations used in this paper:* BGG, bovine gamma globulin; BSA, bovine serum albumin; CS, chondroitin sulfate; CSPG, chondroitin sulfate proteoglycan; Cyt c, cytochrome c; FCS, fetal calf serum; HPLC, high performance liquid chromatography; mAb, monoclonal antibody; MHC, major histocompatibility complex; NP-40, Nonidet P-40; OVA, ovalbumin; PAGE, polyacrylamide gel electrophoresis; PSN, phosphate-buffered saline containing 0.25% NP-40; RAR1g, rabbit anti-rat immunoglobulin; SaCl, *Staphylococcus aureus* Cowan I strain; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; TPCK, L-1-tosylamide-2-phenylethyl-chloromethyl fluoride.

tissues (10, 11) and its specific physical association with a particular membrane glycoprotein is an unusual feature.

To elucidate the possible functional significance of the CSPG in the biology of Ia antigens, we have pursued further biochemical analyses of this molecule. The goal of the studies described in this report was to obtain information on the core protein of the CSPG. We were particularly interested in whether the core protein was structurally related to α , β , or invariant glycoproteins. We present evidence indicating that the core protein of the M_r 46–70 K CSPG isolated from murine spleen cells is an alternately processed form of invariant chain.

Materials and Methods

Radiolabeling and Isolation of the CSPG and its Core Protein. B10.A spleen cells were labeled at a density of 1×10^7 cell/ml for 9–10 h in Dulbecco's modified Eagle's medium, lacking methionine, and supplemented with 10% dialyzed fetal calf serum (FCS), 10 mM Hepes, 1 M glutamine, 5×10^{-5} M 2-mercaptoethanol, and [35 S]methionine (1,000–1,400 Ci/mM; Amersham Corp., Arlington Heights, IL) at 250–500 μ Ci/ml. After labeling, cell pellets were solubilized in 0.5% Nonidet P-40 (NP-40) containing the protease inhibitors phenylmethylsulfonyl fluoride (200 μ g/ml), L-1-tosylamide-2-phenylethyl-chloromethyl ketone (TPCK) (50 μ g/ml), and *N*- α -*p*-tosyl-L-lysine chloromethyl ketone (50 μ g/ml) (Sigma Chemical Co., St. Louis, MO). The detergent lysates were ultracentrifuged at 100,000 *g* for 60 min, and the soluble supernatant was pretreated as previously described (6), and then reacted with anti-I-A^k (10-2.16), anti-Ii (In-1), or control anti-I-A^d (MKD6) monoclonal antibodies. The antigen-antibody complexes were isolated with *Staphylococcus aureus*, Cowan I strain (SaCl) or rabbit anti-rat immunoglobulin/SaCl (RARlg-SaCl) immunoadsorbents (6). Antibody and radiolabeled molecules were eluted from the SaCl pellets by incubations in 0.5 ml of 8 M urea, 0.05 M sodium acetate, 0.15 M sodium chloride, and 0.5% Triton X-100, pH 6 (dissociative DEAE starting buffer). After 16 h at 25°C, particulate material was removed by centrifugation and the soluble supernatant was applied batchwise to the anion exchange resin DEAE-Sephacel (Pharmacia, Inc., Piscataway, NJ), at a ratio of 0.1 ml packed Sephacel per 2.0×10^7 cell equivalents. After incubation for 2 min at 30°C, the unbound (CSPG-depleted) material was collected and diluted 1:1 with deionized water. The DEAE-Sephacel was washed twice with 5.0 ml dissociative DEAE starting buffer and twice with 5.0 ml of DEAE starting buffer without urea. CSPG-enriched material remaining bound to the resin was eluted by incubation of the resin with 0.5 ml of 0.8 M NaCl in 0.05 M sodium acetate and 0.002% Triton X-100, pH 6.0. Radioactivity in the unbound and the eluted fractions was determined, and each sample was dialyzed against a 200-fold excess of 0.005 M Tris-HCl, pH 7.6 for 12 h at 4°C, then for an additional 6 h against fresh buffer. The dialyzed samples were then lyophilized to dryness. The dried unbound fractions were dissolved in 0.5 ml of deionized water and proteins were precipitated by the addition of 3 vol of 95% ethanol containing 1.3% potassium acetate (12). Carrier protein (bovine serum albumin [BSA], ovalbumin [OVA], bovine gamma globulin [BGG] and cytochrome *c* [Cyt *c*]) (Sigma Chemical Co.) was included at a total final concentration of 1.2 mg/ml. The eluted fractions from DEAE-Sephacel were resuspended in 0.1 ml deionized water containing 300 μ g/ml each of BSA, OVA, and IgG and 500 μ g/ml of Cyt *c*, and divided into two equal aliquots. One aliquot was treated with chondroitinase AC (Miles Laboratories, Inc., Elkhart, IN) (1.5 U/ml) and the other was mock digested. In some experiments, chondroitinase ABC (Miles Laboratories, Inc.) was used (see Results). Samples were incubated at 37°C for 2 h and then frozen at –20°C. For analytical or preparative SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis), all samples were adjusted to 0.062 M Tris, 2.0% SDS, 2% 2-mercaptoethanol, 10% glycerol, and 0.001% phenol red, and then boiled for 2.5 min.

Radiolabeling of Invariant Chain. Invariant chain glycoprotein was labeled with [3 H]-

methionine. Labeling was performed as for [³⁵S]methionine (see above) except that [³H]methionine (85 Ci/mM; New England Nuclear, Boston, MA) was used at 200 μ Ci/ml in the labeling medium. The invariant chain was immunoprecipitated with the In-1 monoclonal antibody (mAb), and the 31 kD species was isolated by preparative 11% SDS-PAGE and eluted.

V8 Protease Peptide Mapping of [³⁵S]Methionine-labeled Core Protein. V8 peptide mapping (13) was used to determine if the core protein of the CSPG had any relationship to the α , β , or Ii chain glycoproteins. Samples containing α , β , and invariant chains (unfractionated immunoprecipitates) and samples containing the purified core protein of the CSPG (prepared as detailed above) were electrophoresed separately in the first dimension using a modified Laemmli-Maizel discontinuous SDS/10% PAGE system (14, 15) in 5 \times 120 mm cylindrical gels. The cylindrical gels were then embedded into the top of the second dimension slab gel, using 1.0% low melting point agarose containing 75 μ g/ml *Staphylococcus aureus* V8 protease (Miles Laboratories, Inc.). A modified phosphate-urea gel system (16) was used for the second dimension gels.

The separation gel contained 0.035 M Na₂HPO₄, 0.0152 M NaH₂PO₄, pH 7.1, 6 M urea, 15% acrylamide/bis-acrylamide (37.5:1), 0.1% SDS, 0.02% ammonium persulfate, and 0.05% *N,N,N',N'*-tetramethylethylenediamine (TEMED). The stacking gel contained 0.035 M Na₂HPO₄, 0.152 M NaH₂PO₄, 4.0% acrylamide/bis-acrylamide (37.5:1), 0.1% SDS, 0.02% ammonium persulfate, and 0.18% TEMED. The upper electrode buffer (–) was 0.1 M cacodylic acid, 0.1% SDS, pH 6.0. The lower electrode buffer (+) was 0.035 M Na₂HPO₄, 0.015 M NaH₂PO₄, pH 7.1. Samples were electrophoresed using constant current, at 30 mA per slab gel through the stacking gel at 25°C and at 80 mA per slab gel through the separating gel at 4°C.

Comparative Tryptic Peptide Mapping of Conventional Invariant Chain and CSPG Core Protein by HPLC. Trypsin digestion was performed as previously described (17) with some modifications. In brief, lyophilized samples containing purified invariant chain or purified CSPG core protein were resolubilized in 2.0 ml 0.4 M Tris Cl, pH 8.2 containing 2% SDS and 100 μ g BGG, and were reduced and alkylated. The protein was precipitated with 20% TCA, and the precipitates were washed with ethanol/ether (1:1) and ether to remove the TCA and SDS. The precipitates were resolubilized in 0.5 ml of 0.1 M NH₄HCO₃, pH 8.2, and 0.1 ml TPCK-trypsin (200 μ g/ml in 0.001 N HCl) (Worthington Biochemical Corp., Freehold, NJ) was added. The mixture was incubated for 30 min at 37°C, at which time 10 μ l of 0.4 M NH₄HCO₃, pH 8.2 was added and an additional 0.1 ml TPCK-trypsin, and the incubation was continued for 7 h. The reaction was stopped by the addition of 0.1 ml 0.8 N acetic acid, and the samples were lyophilized. The samples were solubilized in 0.1% trifluoroacetic acid (TFA) (Pierce Chemical Co., Rockford, IL) and chromatographed on a C-18 reverse phase column (Vydac, Hesperia, CA) using a gradient (see Results) of 0–50% acetonitrile (J. T. Baker Chemical Co., Phillipsburg, NJ) in 0.1% TFA and a high performance liquid chromatography (HPLC) system (Waters Associates, Milford, MA).

Reprecipitation of Purified [³⁵S]Methionine-labeled CSPG. [³⁵S]methionine labeled CSPG was isolated by immunoprecipitation with either anti-I-A^k and SaCl or anti-Ii antibodies and RARIg-SaCl. The soluble components of the immunoprecipitates were eluted in dissociative DEAE starting buffer and applied batchwise to DEAE-Sephacel. The unbound fraction was removed and a 0.2 M NaCl, 8 M urea wash of the ion exchange resin was done before elution of the CSPG from the resin by 0.8 M NaCl in 8 M urea. After elution from the resin, the 0.8 M NaCl eluate was diluted to 0.2 M NaCl, 8 M urea and then reapplied to fresh DEAE-Sephacel equilibrated in 0.2 M NaCl, 8 M urea. The resin was again washed, first with 0.2 M NaCl in 8 M urea and then with 0.2 M NaCl before elution with 0.8 M NaCl. The 0.8 M NaCl eluates from the second cycle of ion exchange purification were dialyzed against 0.005 M Tris, pH 7.6, dried, and solubilized in 0.5 ml of deionized water containing 300 μ g/ml BSA, 150 μ g/ml OVA, 300 μ g/ml chondroitin sulfate (CS), and 300 μ g/ml Cyt *c*. Half of each preparation was digested with chondroitinase ABC at a final concentration of 1.5 U/ml for 45 min at 37°C. After digestion, 0.05

ml aliquots of the untreated and chondroitinase-treated samples were added to 0.2 ml of phosphate-buffered saline with 0.25% NP-40 (PSN) containing 200 $\mu\text{g/ml}$ hemoglobin, 2 mg/ml BSA, 2 mg/ml OVA, and 500 $\mu\text{g/ml}$ CS (buffer A). The samples were pretreated with SaCI to reduce nonspecific binding in subsequent experimental immunoprecipitations. Reprecipitation of each sample was attempted by incubating an aliquot of the sample overnight at 4°C with the antibodies indicated in Results. Antigen-antibody complexes were isolated with SaCI (mouse mAb) or RARlg-SaCI (rat mAb) immunoadsorbants. Before use in this experiment, the SaCI was washed three times in buffer A, and was preincubated in PSN containing 1% FCS and 500 $\mu\text{g/ml}$ CS. The second-step immunoprecipitates were washed twice in buffer A, twice in PSN alone, and then analyzed by SDS-PAGE. From each preparation used for the attempted reprecipitation, an aliquot containing radioactivity equivalent to the input used in the immunoprecipitate was adjusted to 0.062 M Tris, 2.0% SDS, 2.0% 2-mercaptoethanol, 10% glycerol, and 0.001% phenol red, and analyzed by SDS-PAGE in parallel with the second-step immunoprecipitates.

Results

Metabolic Labeling and Purification of the Core Protein. The general strategy for biochemical analysis of the core protein of the CSPG was first to isolate immune complexes bearing Ia and invariant glycoproteins with the associated CSPG and then to solubilize the complexes using a buffer that would dissociate the CSPG from the α , β , and invariant chain glycoproteins. After dissociation, the CSPG was physically separated from the proteins and glycoproteins by ion exchange chromatography so that it could be analyzed independently of these other components. The core protein of the CSPG was then isolated by treatment of the proteolygan with a glycosidase to degrade and release the glycosaminoglycan component.

Thus, B10.A spleen cells were biosynthetically labeled with [^{35}S]methionine, and anti-Ia and anti-invariant chain immunoprecipitates were prepared from detergent lysates of the labeled cells. Soluble components from the SaCI-bound immunoprecipitates were eluted in dissociative DEAE starting buffer and applied batchwise to DEAE-Sephacel. Unbound material containing α , β , and invariant chains was collected, and the CSPG bound to the anion exchange resin was recovered by elution with 0.8 M NaCl. The samples were analyzed, concentrated by lyophilization, and divided into two aliquots. One aliquot was treated with the glycosidase chondroitinase ABC, and the other was mock digested. Both samples were then analyzed by SDS-PAGE.

Fig. 1 shows that [^{35}S]methionine-labeled α , β , and invariant proteins are clearly detectable in the unfractionated samples (lanes 3 and 4) and the DEAE-unbound fractions (lanes 7 and 8). In contrast, that fraction of the [^{35}S]methionine-labeled immunoprecipitates which bound to the DEAE-Sephacel consisted almost exclusively of the M_r 46–70 K CSPG molecule (lanes 11 and 12). The amount of radioactivity associated with the M_r 46–70 K component corresponds to ~2–4% of the total amount of labeled material in the immunoprecipitates, demonstrating the degree of purification achieved using the ion exchange procedure.

The SDS gel mobility of the DEAE-bound samples changed dramatically after chondroitinase treatment. There was no detectable radioactive material in the

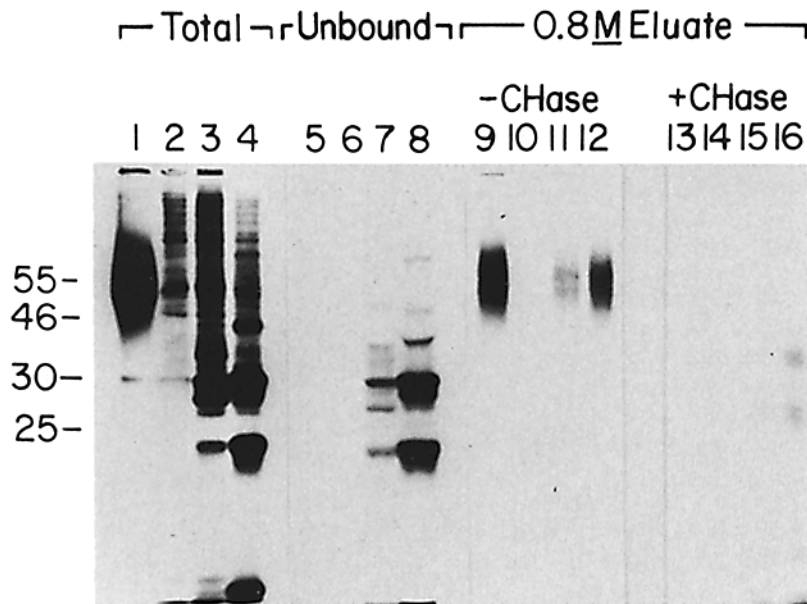


FIGURE 1. SDS-PAGE analysis of immunoprecipitates fractionated by dissociative ion exchange chromatography of immunoprecipitates. B10.A spleen cells were labeled with [35 S]-methionine for 10 h, and immunoprecipitates were prepared from the detergent lysates of labeled cells. 5% of each precipitate was reserved for analysis of unfractionated material (total) and the remaining 95% of each immunoprecipitate was fractionated under dissociative conditions on DEAE-Sephacel. 5% of the DEAE-unbound material was precipitated with 3 vol ethanol. DEAE-bound material was recovered by elution with 0.8 M NaCl (0.8 M eluate). Half of the 0.8 M NaCl eluate was treated with chondroitinase ABC (+CHase) and half was untreated (-CHase). The antibodies used for immunoprecipitation were: anti-I-A^d, used as a negative control (lanes 2, 6, 10, and 14), anti-I-A^k (3, 7, 11, and 15), and anti-Ii (4, 8, 12, and 16). Lanes 1, 5, 9, and 13 represent a $^{35}\text{SO}_4$ -labeled anti-Ii immunoprecipitate used as a positive control for the ion exchange purification and the chondroitinase digestion.

M_r 46–70 K region of the gel, confirming that the methionine-labeled molecule bore chondroitin sulfate glycosaminoglycans. Two lower molecular weight bands, migrating at M_r 38 and 28 K, were detected (Fig. 1, lanes 15 and 16). The finding that there were two protein bands generated after chondroitinase digestion of the M_r 46–70 K proteoglycan suggested that the Ia-associated CSPG either has two distinct core proteins or that the lower molecular weight protein is a partial degradation product of the 38 K species.

To determine if the M_r 38 and 28 K molecules are structurally related to each other, and also to investigate any structural similarities between the core protein(s) of the CSPG and the α , β , and invariant chain glycoproteins, we compared peptides generated by *S. aureus* V8 protease digestion (Cleveland analysis) (13). Anti-Ia and anti-Ii immunoprecipitates were prepared from [35 S]methionine-labeled B10.A spleen cells. 10% of each precipitate was reserved for direct analysis of the α , β , and invariant glycoproteins. The remainder of each precipitate was fractionated on DEAE-Sephacel under dissociative conditions as described in the preceding section, and the eluates from the column were treated with chondroitinase ABC to prepare the core protein. The unfractionated

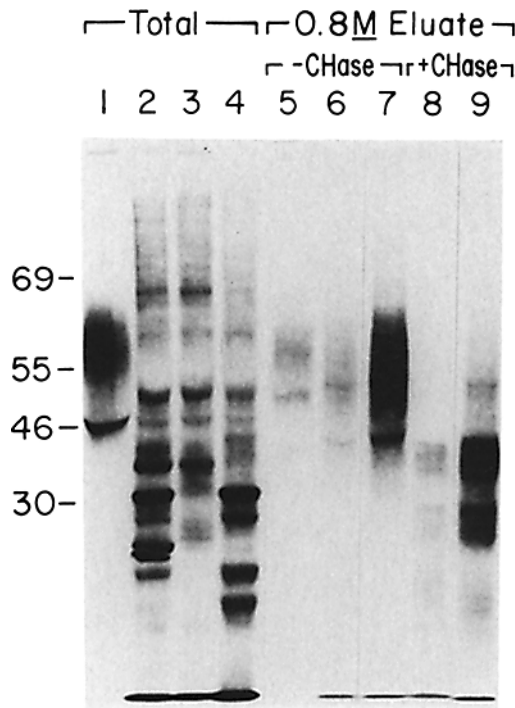


FIGURE 2. One-dimensional SDS-PAGE analysis of CSPG core protein and α , β , and invariant chain. Anti-I-A and anti-I-I immunoprecipitates were prepared from NP-40 detergent lysates of B10.A spleen cells. 10% of the immunoprecipitates were reserved for analysis of unfractionated material (*Total*) and 90% of the immunoprecipitates were fractionated by dissociative ion exchange chromatography on DEAE-Sephacel. Material that bound to the resin was eluted with 0.8 M NaCl (*0.8 M Eluate*) and was either left untreated (*-CHase*) or first treated with chondroitinase ABC (*+CHase*) and analyzed by one-dimensional SDS-PAGE. (Lanes 1 and 5): [^{35}S]sulfate-labeled anti-I-I immunoprecipitate; (2, 6, and 8) [^{35}S]methionine-labeled anti-I-A^s immunoprecipitate; (4, 7, and 9) [^{35}S]methionine-labeled anti-I-I immunoprecipitated. Lane 3 contains [^{35}S]methionine-labeled anti-I-E-immunoprecipitated products but there was not sufficient radioactive material recovered from the eluted fractions of DEAE-Sephacel for further analysis.

immunoprecipitates and the chondroitinase-treated proteoglycan were then electrophoresed by SDS-10% PAGE. 20% of each sample was analyzed by slab gel electrophoresis, and the remainder of each sample was electrophoresed in rod gels for subsequent V8 digestion.

The V8 peptides of the α , β , and invariant proteins in the unfractionated immunoprecipitate were directly compared with those of the core protein of the CSPG by analysis on a single slab gel. The two rod gel segments to be compared were embedded side by side on top of the stacking gel in 1% agarose containing V8 protease, and the chondroitinase-digested core protein(s) and the conventional Ia/Ii glycoproteins were subjected to Cleveland analysis (13) as outlined in Materials and Methods. The V8 peptides were then electrophoresed through a 15% acrylamide gel using a modified phosphate buffer system.

Autoradiographs of the analytical slab gel and the two-dimensional gels are shown in Figs. 2 and 3, respectively. Examination of the one-dimensional slab

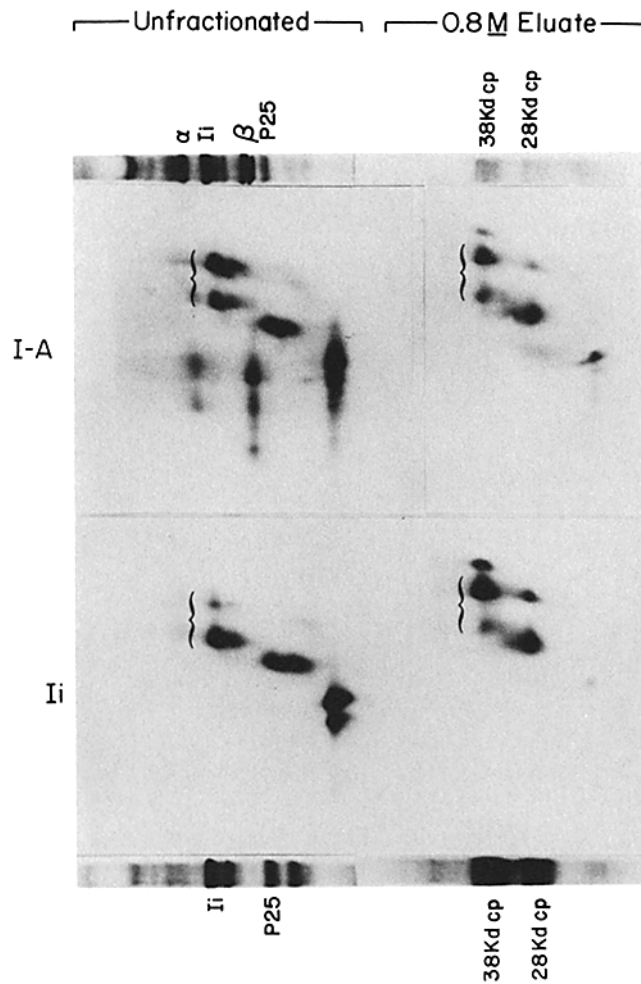


FIGURE 3. Two-dimensional V8 peptide map comparison of the CSPG core protein and α , β , and invariant chain glycoproteins. Unfractionated immunoprecipitates and chondroitinase-treated 0.8 M NaCl eluates from the I-A and Ii preparations (Fig. 2) were subjected to two-dimensional V8 protease peptide mapping (Cleveland analysis). Shown are the autoradiographs of the second dimension gels. One-dimensional SDS gel profiles are shown above (I-A) or below (Ii) autoradiographs of the second dimension gels containing the V8 peptides.

gel (Fig. 2) indicated that chondroitinase digestion of the 0.8 M eluates of each immunoprecipitate yielded two methionine-labeled proteins that migrated at M_r 38 and 28 K, a result similar to that obtained in the previous experiment. Fig. 3 shows the second-dimension slab gels containing the V8 peptides from the anti-I-A and anti-Ii immunoprecipitates. The unfractionated immunoprecipitates are shown at the left and the core protein of the CSPG at the right. The one-dimensional SDS gel profile from each preparation is also shown, to aid identification of the proteins in the V8 digest. Bands corresponding to the α , β , invariant chains, the invariant chain-related "p25" protein, and the M_r 38 and 28 K core proteins, are indicated.

Several observations can be made from this comparative analysis. First, the V8 peptides of the CSPG core proteins precipitated by the I-A-specific and invariant chain-specific reagents were indistinguishable from one another (compare the upper and lower right-hand panels of Fig. 3). This indicates that the same CSPG is isolated with both these reagents. Second, the M_r 38 and 28 K proteins obtained after chondroitinase digestion are structurally similar to each other. Two major peptides were generated by V8 digestion of each protein, and the relative mobilities of the corresponding peptides of the M_r 38 and 28 K proteins were the same. This finding suggests that the 28 K protein may be a proteolytic product of the 38 K molecule. Finally, and most significantly, when we compared the peptides generated from conventional Ia/Ii glycoproteins with those generated from the CSPG core protein (compare left and right panels, Fig. 3), a great deal of similarity was seen between the peptide patterns of the invariant chain and the core protein. Two or three discrete peptides were generated by V8 digestion from both the invariant chain and the core protein (brackets, Fig. 3). Both the intact M_r 38 K core molecule and its V8 peptides migrated as relatively higher molecular weight species than the invariant chain and its peptides. These molecular weight differences are most likely attributable to residual carbohydrate moieties on the core protein. Chondroitinase digestion of proteoglycans leaves at least the internal trisaccharide xylose-galactose-galactose on the core protein. The diffuse gel mobilities of both the M_r 38 and 28 K species are consistent with the presence of variable quantities of residual carbohydrate on the core protein.

The finding that the CSPG core protein and the invariant chain were structurally similar prompted us to explore whether the two molecules might also be antigenically related. An experiment was therefore performed to determine if the CSPG bears the antigenic determinant recognized by the monoclonal anti-invariant antibody, In-I. $^{35}\text{SO}_4$ -labeled CSPG was separated from the Ia and Ii glycoproteins by dissociative ion exchange chromatography. Aliquots of this isolated CSPG were reacted with control, anti-I-A^k or anti-Ii antibodies, and putative antigen-antibody complexes were isolated with SaCl or RARIg-SaCl. Material remaining in the supernatant of the control (anti-I-A^d) immunoprecipitate was reacted with a control rat antibody, and material remaining in the supernatant of the anti-I-A^k immunoprecipitate was reacted with anti-Ii antibody. The results are shown in Fig. 4. The CSPG was not isolated in the anti-I-A^d, anti-I-A^k, or control rat antibody immunoprecipitates (Fig. 4, lanes 2, 3, and 5, respectively), but was isolated in the anti-invariant chain immunoprecipitates (lanes 4 and 6), indicating that the CSPG indeed reacts directly with this antibody.

The experiments shown in Fig. 4 suggested that the Ia-associated CSPG is antigenically related to invariant chain. However, a complication in the interpretation of these experiments was that the CSPG preparation may have been contaminated with small amounts of the M_r 31 K invariant protein, which could potentially reassociate with the CSPG after removal of urea from the samples. This reassociation might have been responsible for the reisolation of the CSPG in anti-Ii immunoprecipitates.

To address this possibility, we repeated the reprecipitation experiment using several modifications in the experimental protocol that we anticipated would

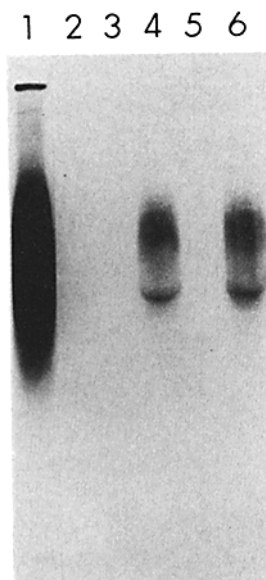


FIGURE 4. [^{35}S]sulfate-labeled CSPG can be reprecipitated with anti-invariant chain antibody. Anti-I-A and anti-Ii immunoprecipitates were prepared from [^{35}S]sulfate-labeled B10.A spleen cells, combined, and fractionated on DEAE-Sephacel under dissociative conditions. Equal aliquots of the DEAE-Sephacel eluate were incubated with a control anti-I-A^d antibody (2), anti-I-A^k (3) or anti-Ii (4). Antigen antibody complexes were isolated with SaCl or RARIG-SaCl. The material remaining in the supernatants of the anti-I-A^d immunoprecipitate was retested with the control rat antibody (5) and material remaining in the supernatant of the anti-I-A^k immunoprecipitate was retested with the anti-Ii antibody (6). Lane 1 shows a portion of the unfractionated combined [^{35}S]sulfate-labeled immunoprecipitates.

decrease or eliminate conventional invariant chain in the CSPG preparations. In addition, we prepared samples labeled with [^{35}S]methionine rather than [^{35}S]sulfate so that presence of the M_r 31 K invariant chain glycoprotein could be directly monitored throughout the purification procedure. The CSPG was isolated from [^{35}S]methionine-labeled spleen cells by immunoprecipitation with anti-I-A^k or anti-Ii antibodies, and was eluted from the SaCl pellets, as before, with dissociative DEAE starting buffer. The CSPG was bound to DEAE-Sephacel and the resin was extensively washed with 0.2 M NaCl in 8 M urea. The CSPG was eluted with 0.8 M NaCl in 8 M urea and, after dilution of the NaCl to 0.2 M, the procedure was repeated. This two-cycle ion exchange chromatography protocol resulted in a CSPG preparation free of invariant chain (see below). Half of each CSPG preparation was then treated with chondroitinase ABC to release the glycosaminoglycan and yield the core protein. The samples were pretreated with SaCl, and aliquots of each sample were reacted with either a control antibody or the antibody originally used to isolate the CSPG. The antibody-antigen complexes were isolated with SaCl or RARIG-SaCl and the immunoprecipitated material was analyzed by SDS-10% PAGE. The results of this experiment are shown in Fig. 5 and a quantitative determination of the radioactivity in the immunoprecipitates is given in Table I.

SDS-PAGE analysis of the 0.8 M NaCl eluates from the ion exchange resin

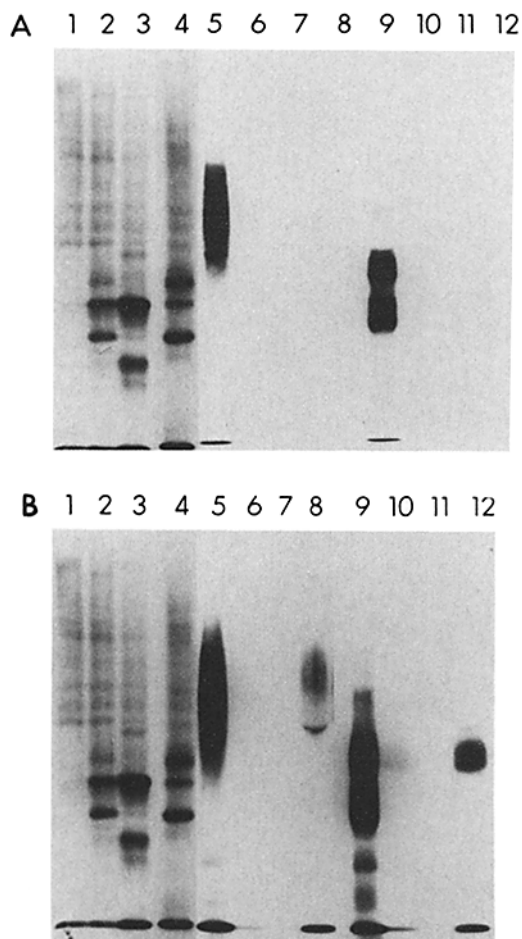


FIGURE 5. Isolation of the twice-purified CSPG derived from B10.BR spleen cells with anti-invariant chain mAb. [^{35}S]methionine-labeled CSPG was prepared from anti-I-A (A) and anti-li (B) immunoprecipitates. The CSPG preparations eluted from the DEAE-Sephacel was reappplied to and eluted from fresh DEAE-Sephacel. The twice-purified CSPG preparations were divided in half and either digested with chondroitinase ABC (lanes 9–12) or mock digested (5–8). Aliquots of the preparations were retested for immunoreactivity with anti-I-A or anti-invariant chain antibodies. Lanes 6–8 and 10–12 in A correspond to samples A–C and D–F in Table I. Lanes 6–8 and 10–12 in B correspond to samples G–I and J–L in Table I. Lanes 5 and 9 correspond to the samples used for the secondary immunoprecipitations. Lanes 1–3 represent anti-I-A^d, anti-I-A^k, and anti-li unfractionated immunoprecipitates, respectively. Lane 4 represents a control unfractionated [^3H]leucine-labeled anti-I-A immunoprecipitate.

(Fig. 5, lanes 5–12) indicates that the modifications in the experimental procedure were sufficient to remove all detectable M_r 31 K invariant chain from the CSPG preparations. When the CSPG preparation from each sample was incubated with SaCl alone, 25–30% of the radioactivity was absorbed to the SaCl (Fig. 5, A and B, lanes 2 and 6, and Table I). After samples had been treated once with SaCl, subsequent nonspecific binding was negligible and specific reactivity with the antibodies could be more rigorously evaluated. In the immunoprecipitates prepared from the I-A sample (Fig. 5A and Table I), virtually no

TABLE I
 Sequential Passage of the CSPG on DEAE-Sephacel before Secondary Immunoprecipitation

First immunoprecipitate	Sample	Chondroitinase treatment	Second antibody	cpm in elute of immunoprecipitate
I-A	A	-	SaCl*	6760
	B	-	I-A ^d	105
	C	-	I-A ^k	165
	D	+	SaCl*	3360
	E	+	I-A ^d	225
	F	+	I-A ^k	150
Ii	G	-	SaCl*	6225
	H	-	PC	90
	I	-	Ii	14,610
	J	+	SaCl*	6765
	K	+	PC	165
	L	+	Ii	13,185

[³⁵S]Methionine-labeled CSPG was prepared from anti-I-A and anti-Ii immunoprecipitates derived from B10.BR spleen cells. The CSPG preparations eluted from DEAE-Sephacel were reapplied to and eluted from fresh DEAE-Sephacel as described in Results. The doubly purified CSPG preparations were digested with chondroitinase ABC (+) or left untreated (-) and then were retested with antibodies as described in Materials and Methods. PC, phosphorylcholine.

* SaCl pretreatment pellets.

radioactivity was isolated with the control anti-I-A^d (lanes 6-8) or the specific anti-I-A^k reagents (lanes 10-12). In the immunoprecipitates prepared from the Ii sample (Fig. 5B), there was no radioactive material isolated with the control rat antibody (Fig. 5B, lanes 7 and 11). In contrast, the anti-invariant chain antibody precipitated large quantities of the CSPG and its M_r 38 K core protein in the total absence of detectable conventional invariant chain (Fig. 5B, lanes 8 and 12, and Table I). Reprecipitation of the M_r 28 K core protein did not occur. These results demonstrate that the In-1 antibody can react directly with both the intact CSPG and the chondroitinase-digested M_r 38 K core protein.

To confirm the specificity of this reaction, supernatants of the anti-I-A^k immunoprecipitates shown in Fig. 5A, lanes 7 and 11 were each divided in half and retested for reaction with control antiphosphorylcholine mAb and anti-Ii mAb. The resulting immunoprecipitates were analyzed by SDS-PAGE. The control antibody gave no reaction (Fig. 6, lanes 2 and 5) but the CSPG and its core protein were again isolated by the anti-invariant chain antibody (Fig. 6, lanes 3 and 6). These results demonstrate the specificity of the reaction of anti-Ii with the CSPG and its core protein.

A control experiment (Fig. 7) demonstrates that lack of reactivity of the CSPG with the I-A^k-specific antibody was not due to denaturation of the antigenic site recognized by the antibody. The stability of the site on I-A _{β} ^k recognized by 10.2.16 has been noted previously (18). In this experiment, the effect of 8 M urea on the antigenic site recognized by the anti-I-A and anti-invariant chain

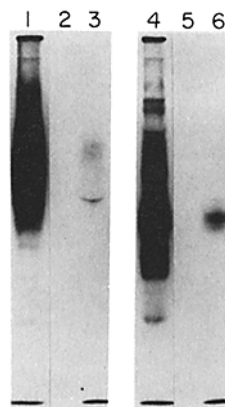


FIGURE 6. Specificity of the reaction of anti-invariant chain antibody with the CSPG and its core protein. Supernatants of the I-A^k immunoprecipitates derived from the CSPG preparation (Fig. 7A, lane 7) on the core protein preparation (Fig. 7A, lane 11) were each divided in half and tested with either control antiphosphorylcholine (lanes 2 and 5) or anti-Ii (3 and 6) mAb. Lanes 1 and 4 show the CSPG and core protein, respectively.

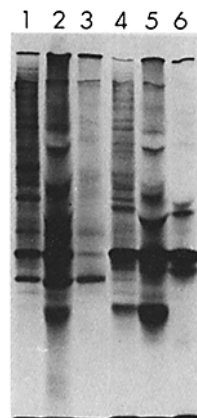


FIGURE 7. Reactivity with anti-I-A and anti-Ii antibodies is maintained after urea treatment. [³⁵S]Methionine-labeled 10-2.16 (lanes 1-3) or In-1 (4-6)-immunoprecipitated material was eluted in urea for 16 h and then fractionated on DEAE-Sephacel. The unbound fractions were dialyzed against phosphate-buffered saline containing 0.1% Triton X-100. Proteins from one aliquot of each sample were precipitated with 3 vol ethanol (lanes 2 and 5), and an equal aliquot was reprecipitated with the antibody originally used for immunoprecipitation (3 and 6). Shown in lanes 1 and 4 are unfractionated 10-2.16 and In-1 immunoprecipitates, respectively.

reagents was evaluated by reprecipitating [³⁵S]methionine-labeled glycoproteins collected after dissociative ion exchange chromatography on DEAE-Sephacel. The unbound fractions containing α , β , and Ii glycoproteins were dialyzed extensively to remove urea, and then reacted with anti-I-A (Fig. 7, lanes 1-3) or anti-Ii (lanes 4-6) antibodies. The resulting precipitates were analyzed by SDS-PAGE. These analyses indicate that both the I-A β chain and the invariant chain retained immunoreactivity with their respective antibodies despite denaturation in urea.

The V8 peptide analysis and immunoprecipitation data indicated that the

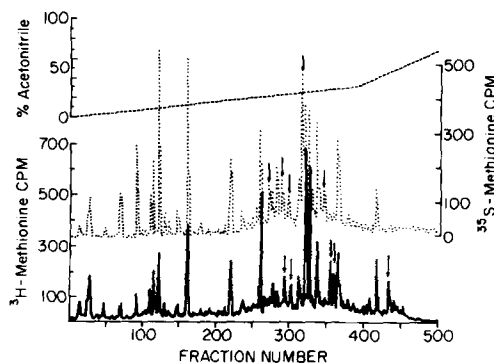


FIGURE 8. Comparative tryptic peptide map of CSPG core protein and invariant chain. [^{35}S]methionine-labeled CSPG core protein and [^3H]methionine-labeled conventional invariant chain were isolated, combined, and digested with trypsin. The tryptic peptides were chromatographed on a C-18 reverse phase HPLC column using a gradient of 0–50% acetonitrile in 0.1% TFA. (· · ·) [^{35}S]Methionine-labeled CSPG core protein peptides; (—) [^3H]methionine-labeled invariant chain peptide; (— — —) acetonitrile gradient. Arrows show peptides that do not coelute.

CSPG core protein and invariant chain were structurally similar and antigenically related. To confirm the structural identity of the core protein and invariant chain, double-label tryptic peptide mapping was performed. [^{35}S]methionine-labeled CSPG was immunoprecipitated with anti-Ii and purified by two cycles of binding to DEAE-Sephacel. The CSPG eluted from the second batch of DEAE-Sephacel was treated with chondroitinase to remove the glycosaminoglycan, yielding [^{35}S]methionine-labeled core protein. [^3H]methionine-labeled conventional M_r 31 K invariant chain was immunoprecipitated with anti-Ii and isolated directly by preparative SDS–11% PAGE. Equal counts per minute of the [^{35}S]methionine-labeled core protein and the [^3H]methionine-labeled invariant chain were combined and digested with trypsin as previously described (17). The resulting tryptic peptides were then separated on a C-18 reverse phase HPLC column, using a gradient of 0–50% acetonitrile in 0.1% TFA. The elution profile of the peptides is shown in Fig. 8. From the sequence of the murine and human invariant chains (19–21), between 9 and 11 methionine-labeled tryptic peptides would have been expected if trypsin digestion had been complete. 11 major and 15 minor peptides are in fact seen for each chain. The larger than expected number of peptides is attributable to partial digestion. 42 of the 52 total peptides coeluted, indicating the nearly complete structural identity between the CSPG core protein and invariant chain. Five peptides from each of the proteins do not coelute (arrows). Because these peptides were derived from molecules that were not treated with neuraminidase, they may bear sialic acid residues on *N*-linked or *O*-linked oligosaccharides. The presence of varying numbers of sialic acid residues on a glycopeptide of the same amino acid sequence can produce HPLC peaks of varying mobility and could account for the lack of coelution of these peptides. The sialic acid residues are more likely to be on the peptides derived from the CSPG core protein and, thus, these peptides would be expected to elute earlier from a reverse phase column than their nonsialated counterparts derived from the invariant chain. Earlier elution of the noncoeluting peptides derived

from the CSPG core protein was in fact the observed result. Another explanation for differences in the peptide elution patterns is variation in posttranslational processing that causes differential tryptic cleavage or affects behavior in reverse phase chromatography.

Discussion

The studies described in this report involve the initial characterization of the core protein of a chondroitin sulfate proteoglycan (CSPG) molecule that specifically associates with murine Ia antigens. We have developed methods for biosynthetically labeling the CSPG with amino acids and for purifying the molecule to be virtually free of other spleen cell components. The purification procedure that proved most successful involved a combination of immunoprecipitation and dissociative anion exchange chromatography. The purified, labeled CSPG was treated with glycosidases to degrade the glycosaminoglycan component and release it from the core protein, so that structural analyses of the core protein could be performed.

One-dimensional SDS-PAGE indicates that the core protein of the CSPG migrates as a doublet of M_r 38 and 28 K. Comparative V8 peptide mapping studies indicate that the 28 K molecule is structurally related to the 38 K molecule. The V8 peptide mapping studies also show that the M_r 38 K core protein yielded a peptide pattern similar to that generated by V8 digestion of invariant chain, and suggested that the two molecules are structurally related. This latter finding raised the intriguing possibility that the core protein of the CSPG might in fact be invariant chain. To investigate this possibility, we evaluated whether the CSPG or its core protein reacts directly with an anti-invariant chain mAb. The reprecipitation data shown in Figs. 4 and 5 indicate that the monoclonal anti-Ii reagent directly bound the intact CSPG and the M_r 38 K core protein but not the 28 K core protein. Tryptic peptide comparison of the M_r 38 K core protein and conventional invariant chain convincingly establish the structural relatedness of the two proteins. Furthermore, in experiments to be presented in a separate report (A. J. Sant, J. Miller, and R. Germain, manuscript in preparation) we have documented that transfection of the murine invariant chain gene into a fibroblastoid cell line leads to expression of the immunoreactive form of the CSPG. Together, our experiments indicate that the CSPG molecule that specifically associates with Ia antigens is an alternately processed form of invariant chain.

A set of invariant chain proteins has been previously demonstrated in mouse and human cells, and it is therefore of interest to consider which of these may serve as a substrate for addition of the glycosaminoglycan. It is established (22) that, although only a single invariant chain gene is present in the murine genome, there are two transcripts from the gene that give rise to M_r 31 and 41 K proteins, both of which bear the epitope recognized by the In-1 monoclonal antibody. In the human system, it has been suggested (21) that multiple transcripts exist and that these could give rise to the other Ii-related proteins routinely coprecipitated with class II molecules. In the murine system, the anti-Ii reagent also coprecipitates smaller polypeptides, including one of M_r ~25 K (p25), which appears to bear some structural relatedness to the 31 K glycoprotein (22, 23). It has not

been firmly established whether these smaller molecules arise from truncated transcripts or are generated by cleavage of a larger protein molecule (p31 or p41). Although some Western blotting experiments have been performed, reactivity of the anti-Ii reagent with p25 is weak at best, and it is not clear whether the p25 no longer bears the In-1 epitope or if the In-1 epitope is simply less stable in the smaller molecule.

The CSPG core proteins that we have recovered have molecular weights of ~38 and 28 K. Based on their residual size heterogeneity, it is likely that the core proteins retain variable amounts of sugar residues. The known cleavage capabilities of chondroitinase indicate that these core proteins retain at least the internal trisaccharide, xylose-galactose-galactose. Therefore, the core polypeptides are actually somewhat smaller than 38 or 28 K. The most likely candidates for CSPG precursors among the Ii family of proteins would thus appear to be the M_r 31 K protein, which carries the In-1 epitope strongly, and the 25 K protein, on which the epitope is represented weakly, if at all. Our data are consistent with this conclusion but do not directly show it.

The implications of our findings should be considered in the context of previous observations regarding the association of invariant chain with the Ia glycoproteins. Sung and Jones (24) and others (25, 26) have presented evidence indicating that invariant chain is exclusively an intracellular component. This was recently confirmed in the human system by Acolla et al. (27), who demonstrated that the M_r 33 K invariant chain and several related invariant proteins are synthesized constitutively in an Ia-negative B cell variant, but that no proteins of the invariant chain family can be detected on the cell surface of the Ia-negative B cell variant or its Ia-positive parent. In addition, the results of Sung and Jones (24) and others (25, 26) suggest that invariant chain interacts only transiently with the intracytoplasmic forms of Ia antigens. Pulse chase labeling and two-dimensional gel analysis performed by Machamer and Cresswell (28) support this hypothesis. They found that, in the human system, invariant chain associates with Ia antigens at the early stages of biosynthesis as an M_r 31 K high-mannose-bearing glycoprotein. With maturation and transport of the α and β glycoproteins to the Golgi apparatus, invariant chain remains associated with the dimer and undergoes coordinate posttranslational modifications including processing of the *N*-linked high mannose oligosaccharides to the complex type and addition of *O*-linked sugars to form "acidic" or "processed" invariant chain. Because neither that M_r 31 K high mannose form nor the 35 K acidic form of invariant chain was detected on the plasma membrane with Ia antigens, their results (28) indicate that, at some stage between these late processing steps and plasma membrane expression of the Ia heterodimer, invariant chain dissociates from the Ia α and β dimer. Similar results have been found recently in the murine system (29), with the exception that the processing of invariant chain was thought not to be completed until after it was dissociated from the α , β dimer.

In other experiments (8), we presented evidence indicating that the CSPG is associated with Ia antigens expressed at the plasma membrane, as well as with an intracellular pool of these glycoproteins. The observation that the core protein of the CSPG is indeed invariant chain leads to speculation that the apparent loss of the invariant chain with maturation and plasma membrane expression of Ia

antigens may not reflect an actual dissociation of this component from the Ia heterodimer but rather a biosynthetic conversion of the M_r 31 or 35 K species to the 46–70 K proteoglycan molecule. In this form, invariant chain would be cotransported with Ia antigens to the plasma membrane.

The finding that invariant chain can be converted to a proteoglycan form which is coexpressed with Ia at the plasma membrane forces a reevaluation of the potential functions of this molecule in the biology of Ia antigens. These potential functions can be organized into three main, nonmutually exclusive categories: biosynthesis/targeting, participation in Ia-mediated cellular interactions, and turnover/recycling. First, invariant chain/CSPG may function in the biosynthesis of Ia by expediting transport of Ia through the intracellular organelles responsible for posttranslational processing, or by targeting Ia to the plasma membrane. Previous studies (24, 28, 29) indicating that invariant chain associates with Ia glycoproteins early in biosynthesis en route to the plasma membrane, and then dissociates from the heterodimer, limited the role of invariant chain to events occurring before expression of Ia at the cell surface. Our finding that the CSPG is associated with cell surface Ia suggests that, if invariant chain is indeed responsible for final transport of Ia to the plasma membrane, it is the CSPG form of invariant chain that mediates this event. In this form, the CSPG might target Ia to specialized regions of the plasma membrane.

Moreover, the association of CSPG with cell surface Ia establishes the latter two categories of potential functions as viable alternatives. Thus, the CSPG could be an integral part of the Ia-oligomer complex on the plasma membrane, and participate directly in Ia-mediated cellular interactions or facilitate such interactions. Alternatively, the CSPG might regulate or direct turnover or recycling of Ia molecules. With regard to recycling, for example, if recycling of Ia is necessary for antigen processing, the CSPG could potentially function to protect the Ia molecules from intracellular degradation.

It is interesting to note that invariant chain shares some unusual structural features with the transferrin receptor, a molecule with a clearly defined function. Based on sequence information derived from cDNA clones, the mouse and human invariant chains (19–21) and transferrin receptor proteins (30, 31) are synthesized without an amino-terminal signal sequence; in contrast to most membrane proteins, these proteins appear to be oriented with their amino termini on the cytoplasmic side of the membrane. In addition, both transferrin receptor (32) and invariant chain (S. Simonis and S. E. Cullen, manuscript submitted) can be covalently fatty acylated. Finally, just as invariant chain can be alternately processed to a proteoglycan form, it has recently been found (33) that the transferrin receptor can be alternately processed to a heparin sulfate proteoglycan form which remains reactive with anti-transferrin receptor antibody and can still bind transferrin. The involvement of transferrin receptor in the intracellular transport of transferrin, and the general structural similarities between transferrin receptor and invariant chain, lead to speculation that an analogous intracellular transport function exists for invariant chain.

An unresolved issue is whether there is an obligatory role of invariant chain/CSPG in the normal biology of Ia glycoproteins. To evaluate the necessity of invariant chain in normal Ia maturation and plasma membrane expression, it

should be possible to transfect cells that are totally deficient in invariant chain with α and β chain genes and determine if the corresponding gene products are expressed at the plasma membrane. In addition, comparison of cells transfected with α , β , and invariant chain genes with those transfected with only α and β chain genes, or a comparison of invariant chain nonexpressor mutants with parental cells, should allow an assessment of the role of the invariant chain/CSPG in Ia processing and turnover/recycling and in antigen presentation. Approaches such as these should resolve some of the issues raised and elucidate the role of the various forms of invariant chain in the biology of the Ia molecules.

Summary

The murine Ia-associated chondroitin sulfate proteoglycan (CSPG) was studied both biochemically and immunochemically to determine the nature of its core protein. Chondroitinase ABC or chondroitinase AC treatment of the CSPG digested the chondroitin sulfate glycosaminoglycan, yielding a core protein that migrated with an apparent molecular weight of 38,000. Comparative V8 protease digestion of the CSPG core protein and conventional invariant glycoproteins yielded homologous peptides, indicating that the core protein and invariant chain were structurally similar. The purified CSPG and its core protein were both shown to react directly with the monoclonal anti-invariant chain antibody, In-1. Comparative tryptic peptide analysis by high performance liquid chromatography demonstrated coelution of the majority of the peptides from the invariant chain and the CSPG core protein. Collectively, these results indicate that the CSPG is an alternatively processed form of invariant chain.

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References

1. Jones, P., D. B. Murphy, D. Hewgill, and H. O. McDevitt. 1978. Detection of a common polypeptide chain in I-A and I-E subregion immunoprecipitates. *Immunochemistry*. 15:51.
2. Quill, H., and B. D. Schwartz. 1984. Invariant proteins associated with guinea pig Ia antigens. *Mol. Immunol.* 21:1333.
3. Frelinger, J. G., L. Hood, and P. J. Wettstein. 1981. Analysis of RT1 products using two dimensional polyacrylamide gels. *Transplant. Proc.* 13:1360.
4. Charron, D. J., and H. O. McDevitt. 1979. Analysis of HLA-D region associated molecules with monoclonal antibody. *Proc. Natl. Acad. Sci. USA.* 76:6567.
5. Shackelford, D. A., and J. L. Strominger. 1980. Demonstration of structural polymorphism among HLA-DR light chains by two dimensional gel electrophoresis. *J. Exp. Med.* 151:144.
6. Sant, A. J., B. D. Schwartz, and S. E. Cullen. 1983. Identification of a new component in the murine Ia molecular complex. *J. Exp. Med.* 158:1979.
7. Sant, A. J., S. E. Cullen, and B. D. Schwartz. 1984. Identification of a sulfate-bearing molecule associated with HLA class II antigens. *Proc. Natl. Acad. Sci. USA.* 81:1534.

8. Sant, A. J., S. E. Cullen, and B. D. Schwartz. 1985. Biosynthetic relationships of the chondroitin sulfate proteoglycan with Ia and invariant chain glycoproteins. *J. Immunol.* 135:416.
9. Sant, A. J., B. D. Schwartz, and S. E. Cullen. 1985. Cellular distribution of the Ia-associated chondroitin sulfate proteoglycan. *J. Immunol.* 135:408.
10. Roden, L. 1980. Structure and metabolism of connective tissue proteoglycans. In *Biochemistry of Glycoproteins and Proteoglycans*. W. A. Lennarz, editor. Plenum Publishing Corp. New York. 267-371.
11. Hascall, V. C., and G. K. Hascall. 1981. Proteoglycans. In *Cell Biology of the Extracellular Matrix*. E. Hay, editor. Plenum Publishing Corp., New York. 39-63.
12. Kimata, K., M. Okayama, A. Oohira, and S. Suzuki. 1974. Heterogeneity of proteo-chondroitin sulfates produced by chondrocytes at different stages of cytodifferentiation. *J. Biol. Chem.* 249:1646.
13. Cleveland, D. W., S. G. Fisher, M. W. Krischner, and U. K. Lammler. 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J. Biol. Chem.* 252:1102.
14. Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of the bacteriophage T4. *Nature (Lond.)*. 227:680.
15. Maizel, J. V., Jr. 1971. Polyacrylamide gel electrophoresis of viral proteins. *Methods Virol.* 5:179.
16. Pugsley, A. T., and C. A. Schnaitman. 1979. Factors affecting the electrophoretic mobility of the major outer membrane proteins of *Escherichia coli* in polyacrylamide gels. *Biochim. Biophys. Acta.* 581:163.
17. Abruzzini, L. N. K. F., and B. D. Schwartz. 1982. Tentative assignment of alleles for guinea pig Ia antigens. I. Ia 3,5 and Ia 4,5 share structural homology expected for alleles. *J. Immunol.* 128:2682.
18. Kupinski, J. M., M. L. Plunkett, and J. H. Freed. 1983. Assignment of antigenic determinants to separated I-A^k chains. *J. Immunol.* 130:2277.
19. Singer, P. A., W. Lauer, Z. Dembic, W. E. Mayer, J. Lipp, N. Koch, G. Hammerling, J. Klein, and B. Dobberstein. 1984. Structure of the murine Ia-associated invariant (Ii) chain as deduced from a cDNA clone. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:873.
20. Strubin, M., B. Mach, and E. O. Long. 1984. The complete sequence of the mRNA for the HLA-DR-associated invariant chain reveals a polypeptide with an unusual transmembrane polarity. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:869.
21. Claesson, L., D. Larhammar, L. Raste, and P. A. Peterson. 1983. cDNA clone for the human invariant γ chain of class II histocompatibility antigens and its implications for the protein structure. *Proc. Natl. Acad. Sci. USA.* 80:7395.
22. Yamamoto, K., N. Koch, M. Steinmetz, and G. J. Hammerling. 1985. One gene encodes two distinct Ia-associated invariant chains. *J. Immunol.* 134:3461.
23. Zecher, R., W. Ballhausen, K. Reske, D. Linder, M. Schluter, and S. Stirm. 1984. The invariant chains of mouse class II antigens: biochemical properties and molecular relationship. *Eur. J. Immunol.* 14:511.
24. Sung, E., and P. P. Jones. 1981. The invariant chain of murine Ia antigens: its glycosylation, abundance and subcellular localization. *Mol. Immunol.* 18:889.
25. Owen, M. J., A. M. Kissonerghis, H. F. Lodish, and M. J. Crumpton. 1981. Biosynthesis and maturation of HLA-DR antigens in vivo. *J. Biol. Chem.* 256:8987.
26. Moosic, J. P., E. Sung, A. Nilson, P. P. Jones, and D. McKean. 1982. The selective solubilization of different murine splenocyte membrane fractions with Lubrol WX and Triton X-100 distinguishes two forms of Ia antigens. *J. Biol. Chem.* 257:9684.
27. Acolla, R. S., G. Carra, F. Buchegger, S. Carrel, and J. P. Mach. 1985. The human Ia-associated invariant chain is synthesized in Ia-negative B cell variants and is not

- expressed on the cell surface of both Ia-negative and Ia-positive parental cells. *J. Immunol.* 134:3265.
28. Machamer, C. E., and P. Cresswell. 1982. Biosynthesis and glycosylation of the invariant chain associated with HLA-DR antigens. *J. Immunol.* 129:2564.
 29. Holt, G. D., S. J. Sweidler, J. H. Freed, and G. W. Hart. 1985. Murine Ia-associated invariant chain's processing to complex polysaccharide forms and its dissociation from the I-A^K complex. *J. Immunol.* 135:399.
 30. Stearne, P. A., G. A. Pietersz, and J. W. Goding. 1985. cDNA cloning of the murine transferrin receptor: sequence of transmembrane and adjacent regions. *J. Immunol.* 134:3474.
 31. Schneider, S., M. J. Owen, D. Bonville, and J. G. Williams. 1984. Primary structure of human transferrin receptor deduced from the mRNA sequence. *Nature (Lond.)* 311:675.
 32. Omari, M. B., and I. S. Trowbridge. 1981. Covalent binding of fatty acid to the transferrin receptor in cultured human cells. *J. Biol. Chem.* 256:4715.
 33. Fransson, L.-A., I. Carlstedt, L. Coster, and A. Malmstrom. 1984. Binding of transferrin to the core protein of fibroblast proteoglycan sulfate. *Proc. Natl. Acad. Sci. USA.* 81:5657.