THE HUMAN INVARIANT CHAIN IS THE CORE PROTEIN OF THE HUMAN CLASS II–ASSOCIATED PROTEOGLYCAN

By KAREN S. GIACOLETTO,* ANDREA J. SANT,* CHRIS BONO,*‡ JOHN GORKA,*‡ DEIRDRE M. O'SULLIVAN,§ VITO QUARANTA,§ AND BENJAMIN D. SCHWARTZ*‡

*From the *Departments of Medicine and Microbiology and Immunology and the ‡Howard Hughes Medical Institute, Washington University School of Medicine, St. Louis, Missouri 63110; and §Department of Immunology, Research Institute of Scripps Clinic, La Jolla, California 92037*

The major histocompatibility complex (MHC) class II molecules are critical to the generation of the immune response. These molecules consist of an α chain of ~34,000 M_r and a β chain of ~25,000 M_r which are encoded by the MHC. Helper T cells are known to recognize processed foreign antigen in the context of this $\alpha\beta$ heterodimer. The $\alpha\beta$ heterodimer has been found to be associated with several non-MHC–encoded molecules during its biosynthesis and/or expression, but the role of these molecules in the biology of the class II molecules is largely unknown. In humans, several molecules have been described. These molecules include the γ_1 or invariant chain, and the related species γ_2, γ_3, p41, and p25 (1–5). In addition, the $\alpha\beta$ heterodimer has been shown to be associated with a 40,000–70,000 M_r chondroitin-4-sulfate proteoglycan molecule (6).

Recent studies from our laboratory (7) in mice showed that the core protein of the murine Ia-associated proteoglycan was the murine invariant chain, and thus established the proteoglycan as an alternatively processed form of invariant chain that was associated with class II molecules. In this report, we present data indicating that the human invariant chain and some of its related components are the core protein(s) of the human class II–associated proteoglycan. In addition, we demonstrate that virtually all of the class II–associated proteoglycan is present at the cell surface.

Materials and Methods

Cells. The DR5 homozygous cell line, Swei (HLA-A29, A29, B40, B40, DR5, DR5, DRw52, DRw52, DQw3, DQw3) originally obtained from John Hansen (Fred Hutchinson Cancer Research Center, Seattle, WA) was used as the source of the proteoglycan in these studies (8). The Swei cells were maintained in RPMI 1640 containing 10% dialyzed FCS.

Antibodies. 1800D2.4 is a murine anti-DR monoclonal antibody produced in our laboratory. Leu-10 is a murine anti-DQ antibody purchased from Becton, Dickenson & Co., (Mountain View, CA) (9). VIC-Y1, a murine anti–human invariant chain antibody has been described previously (5). C351 is a rabbit antiserum produced against a synthetic

This study was supported in part by grants AI-18925, AI-15322, and AI-15353 from the U.S. Public Health Service. Dr. Quaranta is a Scholar of the Leukemia Society. Dr. Sant is currently with the Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Building 10, Room 11N-311, Bethesda, MD 20892.

peptide corresponding to the C-terminal region (residues 192–211) of human invariant chain.[1] D1C10 is an irrelevant murine monoclonal antibody used as a control. Rabbit anti-mouse immunoglobulin (RαMIg)[2] was purchased from Accurate Chemical & Scientific Corp. (Westbury, NY.)

Biosynthetic Labeling and Solubilization. Biosynthetic labeling with [^{3}H]leucine, $^{35}SO_4$, [^{35}S]methionine, and [^{3}H]methionine was done as previously described (6, 7). In brief, cells were labeled at a density of 2×10^6 cells/ml for 5 h at 37°C in precursor-deficient media containing 250–500 μCi of the appropriate radiolabeled precursor. After washing, cells were solubilized either in PBS containing 0.5% NP-40 or in 0.05 M sodium acetate, 0.15 M NaCl, 0.5% Triton X-100, pH 6.0 (associative DEAE starting buffer), containing protease inhibitors (6, 7). The cell lysates were ultracentrifuged at 100,000 *g* for 60 min to remove insoluble debris.

Purification of the Class II–associated Proteoglycan. Under associative buffer conditions, the associative DEAE-buffer lysate was applied in batch to the anion-exchange resin DEAE-Sephacel (Pharmacia Inc., Piscataway, NJ) at a ratio of 0.1 ml of packed Sephacel per 10^8 cell equivalents. Unbound material was collected, and the DEAE-Sephacel was washed with 0.2 M NaCl, 0.05 M sodium acetate, 0.5% Triton X-100, pH 6.0. Bound material was eluted with 0.8 M NaCl, 0.05 M sodium acetate, 0.002% Triton X-100 (DEAE elution buffer). After elution, the eluate was adjusted to 0.2 M NaCl, pH 7.4.

Immunoprecipitation. Detergent lysates or DEAE-purified eluates were precleared (6, 7) and then reacted with monoclonal antibodies, or rabbit antiserum. Monoclonal antibody–antigen complexes were pelleted using protein A–Sepharose (Pharmacia, Inc.) which had been prearmed with RαMIg (RαMIg–protein A). Rabbit antiserum–antigen complexes were pelleted with protein A–Sepharose.

Purification of the Class II–associated Proteoglycan under Dissociative Conditions. Antibody and radiolabeled molecules were eluted from the immunoprecipitates using DEAE-associative starting buffer containing 8 M urea (DEAE-dissociative starting buffer), and the eluates were applied batchwise to fresh DEAE-Sephacel. Unbound material was collected, and the DEAE-Sephacel was washed in DEAE-dissociative buffer containing 0.2 M NaCl, Material bound to the DEAE-Sephacel was eluted with DEAE elution buffer.

Chondroitinase Treatment of the Class II-associated Proteoglycan. The DEAE elution buffer containing the doubly purified chondroitin sulfate proteoglycan (CSPG) was diluted with three volumes of chondroitinase buffer (0.05 M Tris, pH 6.0, containing 0.01% BSA) and divided into two aliquots. One aliquot was treated with chondroitinase AC (Miles Laboratories, Inc., Elkhart, IN) (1.5 U/ml) to yield the core protein of the proteoglycan, and the other was mock digested. In some experiments, chondroitinase ABC (Miles Laboratories, Inc.) was used in place of chondroitinase AC. Samples were incubated at 37°C for 2 h. The core protein or mock-digested proteoglycan was precipitated by the addition of TCA to a final concentration of 20% (wt/vol).

SDS-PAGE. For analytical or preparative SDS-PAGE, immunoprecipitates were eluted in or samples were adjusted to 0.062 M Tris, 2.0% SDS, 2% 2-ME, 10% glycerol, and 0.001% phenol red, and then boiled for 2.5 min. Samples were electrophoresed through a 1-cm 4% stacking gel and a 11.5-cm 11% (except where indicated) running gel using a modification of the Laemmli-Maizel system (10, 11). Gels were processed for autoradiography.

Trypsin Digestion. The bands of interest in the SDS-PAGE slab gel were localized by autoradiography. The pieces of gel containing the bands were excised, reswollen in 30% methanol/10% acetic acid, washed in distilled water, and then placed in 0.5 ml of 0.1 M NH_4HCO_3, pH 8.2, containing 0.1 ml of *N*-tosyl-L-phenylalanine chloromethyl ketone

[1] O'Sullivan, D. M., C. Wong, R. A. Houghten, and V. Quaranta. Identification of invariant chain–related components in Ia oligomers by antipeptide antisera. Manuscript submitted for publication.

[2] *Abbreviations used in this paper:* CSPG, chondroitin-4-sulfate proteoglycan; NRS, normal rabbit serum; RαMIg, rabbit anti-mouse immunoglobulin; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone.

(TPCK)-trypsin (200 μg/ml) in 0.001 N HCl) (Worthington Biochemical Corp., Freehold, NJ). The mixture was incubated for 30 min at 37°C, at which time 10 μl of 0.4 M NH_4HCO_3, pH 8.2, and an additional 0.1 ml of TPCK-trypsin was added, and the incubation was continued overnight. The reaction was stopped by the addition of 0.1 ml of 0.8 N acetic acid. The gel pieces were removed by centrifugation, and the eluates were 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 trifluoroacetic acid (Pierce Chemical Co., Rockford, IL) and a HPLC system (Waters Associates, Milford, MA).

Reprecipitation of the CSPG and Its Core Protein. The mock-digested and chondroitinase-treated doubly purified CSPG containing the CSPG and its core protein respectively were each pretreated by immunoprecipitation with D1C10 and RαMIg–protein A. Aliquots of the pretreated samples were reacted with D1C10, 1800D2.4, VIC-Y1, C351, or normal rabbit serum (NRS), and immunoprecipitation was accomplished using RαMIg–protein A, or protein A as appropriate. The immunoprecipitates were analyzed by SDS-PAGE using a 11% running gel.

Two-dimensional Gel Electrophoresis. The mock-digested, doubly purified CSPG and the chondroitinase-treated, doubly purified core protein were chromatographed on Sephadex G-25 in IEF elution buffer (8 M urea, 5% 2-ME, 2% ampholytes, pH 3.5–10, [LKB Products, Gaithersburg, MD] and 0.1% NP-40). The samples were then analyzed by two-dimensional gel electrophoresis as previously described (6, 12).

Reaction of Antibody with Intact Cells. Swei cells were labeled for 5 h with [^{3}H]leucine or $^{35}SO_4$. After labeling, viable cells were purified by centrifugation through Ficoll-Hypaque, washed, and divided into two groups. One group of cells was incubated with antibody for 30 min at 4°C, washed three times in PBS containing 1% dialyzed FCS at 4°C, and then solubilized in 0.5% NP-40 containing a fivefold excess of unlabeled Swei cell extract. The unlabeled extract was added to block any available binding sites remaining on the cell-bound antibodies. The second group of cells was solubilized immediately with 0.5% NP-40, and aliquots of the detergent lysate were incubated with antibody for 30 min at 4°C. Antigen-antibody complexes were then isolated using RαMIg bound to *Staphylococcus aureus* Cowan I strain. Immune complexes were dissociated with SDS and were analyzed by SDS-PAGE.

Chondroitinase Treatment of Intact Cells. Swei cells were radiolabeled with $^{35}SO_4$ or [^{3}H]leucine for 3 h, washed, and then resuspended in 0.15 M NaCl, 0.005 M Tris, pH 7.4, containing 0.01% BSA and 0.02% sodium azide. The sodium azide was added to diminish turnover and/or endocytosis of the CSPG (13). Chrondroitinase AC (1.5 U/ml) was added to half of each preparation, and the samples were incubated at 37°C for 30 min. The cells were washed three times in PBS. Cell viability was >98% as judged by trypan blue exclusion. The cells were solubilized in PBS containing 0.5% NP-40. The lysates were ultracentrifuged and pretreated, and immunoprecipitates were prepared and analyzed by SDS-PAGE on 11% slab gels as described above.

Results

The strategy we used to characterize the core protein of the CSPG associated with human class II molecules was adapted (Fig. 1) from the one successfully employed in the murine system (7). In brief, [^{35}S]methionine-radiolabeled proteoglycans and their associated molecules were purified from lysates of Swei cells by their ability to be bound by DEAE-Sephacel. This first purification was performed under associative conditions to maintain the αβγ-CSPG complex as a unit. Unbound material was collected, and then the DEAE-bound material that contained the αβγ-CSPG complex was eluted with 0.8 M NaCl. After dilution, the αβγ-CSPG complex was isolated from other proteoglycans in the eluate by specific immunoprecipitation with the murine anti–human invariant chain monoclonal antibody VIC-Y1 (5). The CSPG was then dissociated with other compo-

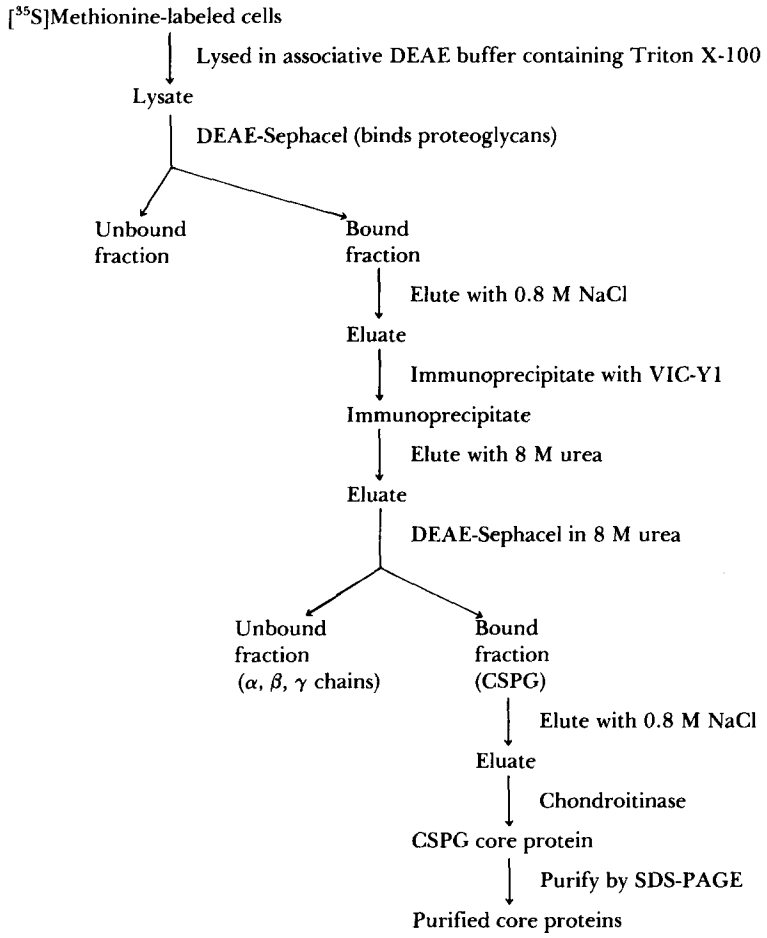

FIGURE 1. Strategy for characterization of the core protein.

nents in the complex by 8 M urea treatment, and purified by DEAE-Sephacel chromatography under dissociative conditions to preclude its reassociation with the α, β, or γ chains. The unbound fraction containing these chains was collected and the bound fraction containing the CSPG was again eluted by 0.8 M NaCl. After dilution, the purified CSPG was treated with the chondroitin sulfate glycosaminoglycan–specific glycosidase, chondroitinase AC, to generate the CSPG core protein(s). The core proteins were purified by SDS-PAGE and compared with the family of invariant chains by a number of biochemical and immunochemical analyses.

Fig. 2 shows the SDS-PAGE analysis of the doubly purified [^{35}S]methionine-labeled CSPG before chondroitinase treatment (lane *4*). The CSPG migrates as a broad band with an M_r of 40,000–70,000. Particularly notable is the absence of any bands corresponding to the α, β, or γ chains, similar to the finding in the murine system (7). The doubly purified CSPG represents ~19.5% of the radioactivity immunoprecipitated from the associative DEAE-Sephacel–bound fraction, 0.25% of the radioactivity in the associative DEAE-Sephacel–bound fraction

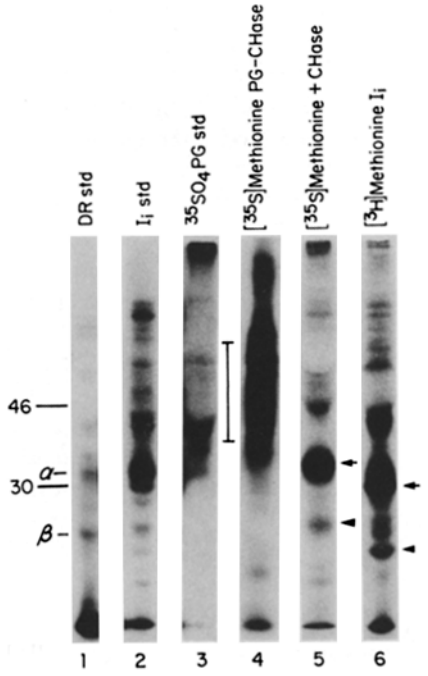

FIGURE 2. Isolation of the twice-purified CSPG core protein. Swei cells were labeled with [^{35}S]methionine and solubilized in DEAE-associative starting buffer. The CSPG was purified with class II molecules by associative anion-exchange chromatography and immunoprecipitation using the anti-human invariant chain monoclonal antibody VIC-Y1, and then alone by dissociative anion-exchange chromatography. An aliquot of the twice-purified CSPG was mock digested (*[^{35}S]Methionine PG-CHase*), and the remainder was treated with chondroitinase (*[^{35}S]Methionine + CHase*). Other Swei cells were labeled with [^{3}H]methionine and solubilized in NP-40, and the lysate reacted directly with VIC-Y1 (*[^{3}H]Methionine I_i*). All samples were subjected to SDS-11% PAGE. [^{35}S]Methionine-labeled anti-DR (lane *1*) and VIC-Y1 (lane *2*) immunoprecipitates of the unbound associative fraction and a $^{35}SO_4$-labeled VIC-Y1 immunoprecipitate showing the CSPG (*bracket*) are shown for comparison. The mock-digested [^{35}S]methionine-labeled CSPG (lane *4*), the chondroitinase-digested [^{35}S]methionine-labeled CSPG core proteins (lane *5*), and the [^{3}H]methionine-labeled invariant chain proteins (lane *6*) are shown. The *arrows* and *arrowheads* next to lanes *5* and *6* indicate the bands taken for comparative tryptic peptide mapping. Abbreviation: CHase, chondroitinase.

itself, and 0.014% of the radioactivity in the whole cell lysate. This degree of purification demonstrates the power of this simple three-step procedure.

The SDS-PAGE profile of the core protein (lane *5*) obtained after chondroitinase treatment of the CSPG differs dramatically from that of the CSPG. The broad band in the M_r 40,000–70,000 region of the gel is no longer seen, demonstrating the efficacy of the chondroitinase treatment. Instead, a series of lower-M_r [^{35}S]methionine-labeled proteins are seen. The most intense of these bands migrates with an M_r of ~38,000 (lane *5*, *arrow*). Less intense bands migrate with M_r of ~46,000 and 28,000 (lane *5*, *arrowhead*). The finding of three protein bands generated after chondroitinase digestion of the CSPG suggested that the CSPG could have as many as three core proteins, or that the lower-M_r core proteins were partial degradation products of the larger.

To determine in a preliminary fashion if the generated core protein compo-

nents had any relationship to the members of the family of invariant chain components, an anti–invariant chain immunoprecipitate prepared from an NP-40 lysate of [^{3}H]methionine-labeled Swei cells was analyzed by SDS-PAGE in a parallel lane (lane *6*). A series of bands is again seen. The most intense band migrates with an M_r of ~31,000 (lane *6*, *arrow*); less intense bands migrate with M_r of ~41,000 and ~25,000 (lane *6*, *arrowhead*). These bands correspond to the γ_1, p41, and p25 members of the family of invariant chain–related proteins. Although the CSPG core proteins (lane *5*) were larger than the invariant chain proteins (lane *6*), their periodicity was similar, suggesting a possible relationship between the two sets of proteins.

This relationship was further explored by comparative two-dimensional gel analysis of the CSPG, its core proteins, and the invariant chain proteins (Fig. 3). The CSPG migrates as two major species (Fig. 3*A*), similar to the pattern seen for the murine CSPG (14). The major component (*bracket*) was detected as a vertical band of fairly homogeneous pI of ~6.1, and M_r of ~40,000–70,000. The second major component (*brace*) is seen as a diagonal spot of more heterogeneous pI (~4.2–5.0) and M_r of ~50,000–60,000. A series of light spots (*arrows*) of pI 4.6–5.8 and M_r ~38,000–40,000 is also observed. The intense high-M_r acidic spots have not been identified, but for the most part they are unaffected by chondroitinase treatment (compare with Fig. 3*B*). Of note again is the absence of any detectable conventional invariant chain components in this doubly purified CSPG preparation.

The two-dimensional gel pattern of the core proteins (Fig. 3*B*) released from the CSPG by chondroitinase treatment is quite distinct from that of the CSPG. The two major components of the CSPG are no longer seen. The most intense set of spots corresponding to the core proteins (*bracket*) is seen in the 38,000 M_r range of the gel, across a heterogeneous pI range (4.5–7.2). The most acidic of these spots (*arrows*) correspond exactly to the series of light spots seen in the two-dimensional gel analysis of the CSPG (Fig. 3*A*, *arrows*) but are now more intense. A second set of spots (curly bracket) is seen in the 28,000 M_r range of the gel. The most intense of these migrates in the pI range of 4.0–5.0. No readily discernible set of core proteins in the M_r 46,000 range was seen.

Two-dimensional gel analysis of the anti–invariant chain immunoprecipitate (Fig. 3*C*) demonstrated the previously observed pattern (3, 5). Comparison of the core proteins (*B*) with the invariant chain proteins showed that the 38,000 M_r set of core proteins (*B*, *bracket*) corresponded to the 31,000 M_r set of invariant chain proteins (Fig. 3*C*, *bracket*), but migrated at a more acidic pI. The 28,000 M_r set of core proteins (*B*, *brace*) shows little obvious correspondence to the 25,000 M_r set of invariant chain proteins (*C*, *brace* and *arrowheads*), and migrates in a more acidic pI range.

Although the two-dimensional gel analysis suggested a possible correspondence between the CSPG core proteins and invariant chain proteins, the results were far from conclusive. We therefore compared these sets of proteins by double-label tryptic peptide analysis. Pieces of gel containing the 38,000 M_r species of core protein (Fig. 2, lane *5*, *arrow*) and the 31,000 M_r species of invariant chain (Fig. 2, lane *6*, *arrow*) were excised, and combined. Similarly, pieces of gel containing the 28,000 M_r species of core protein and the 25,000 M_r species of

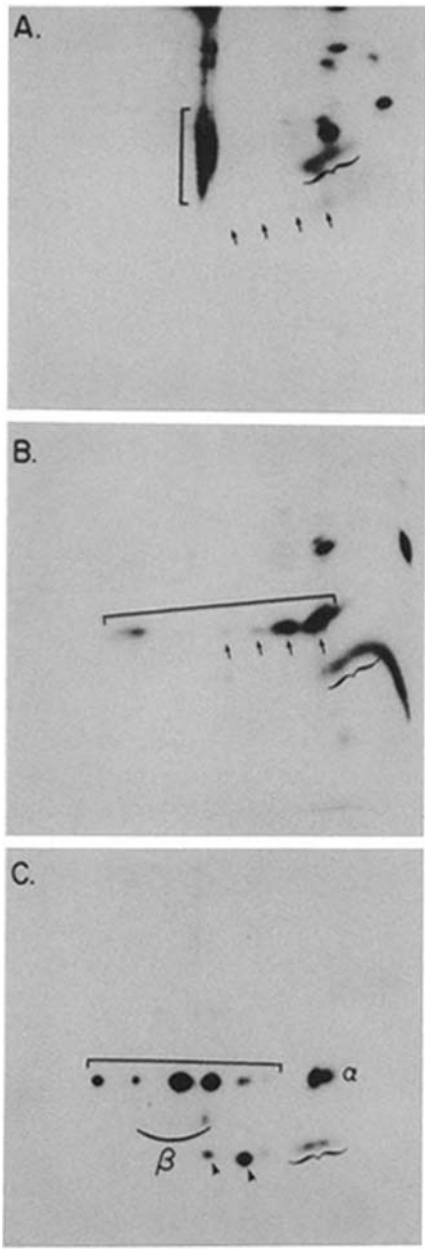

FIGURE 3. Two-dimensional gel comparison of the CSPG, its core proteins, and invariant chain proteins. Aliquots of the material analyzed in lanes *4*, *5*, and *6* by one-dimensional gel electrophoresis were also analyzed by two-dimensional gel electrophoresis. The CSPG (*A*) migrates as two major components (*bracket* and *brace*), and several minor components (*arrows*). After chondroitinase digestion (*B*), the two major components are no longer seen. Instead, the core protein migrates as a series of spots of ~38,000 M_r (*bracket*), the most acidic of which (*arrows*) correspond exactly to the four components (*arrows*) seen in *A*. In addition, a 28,000 M_r acidic core protein is seen (*B*, *brace*). The invariant chain proteins (*C*) migrate as a major series of spots of ~31,000 M_r two 25,000 M_r spots (*arrowheads*) and an acidic 25,000 M_r spot (*brace*). The α and β chains coprecipitated with the invariant chain antibody are labeled.

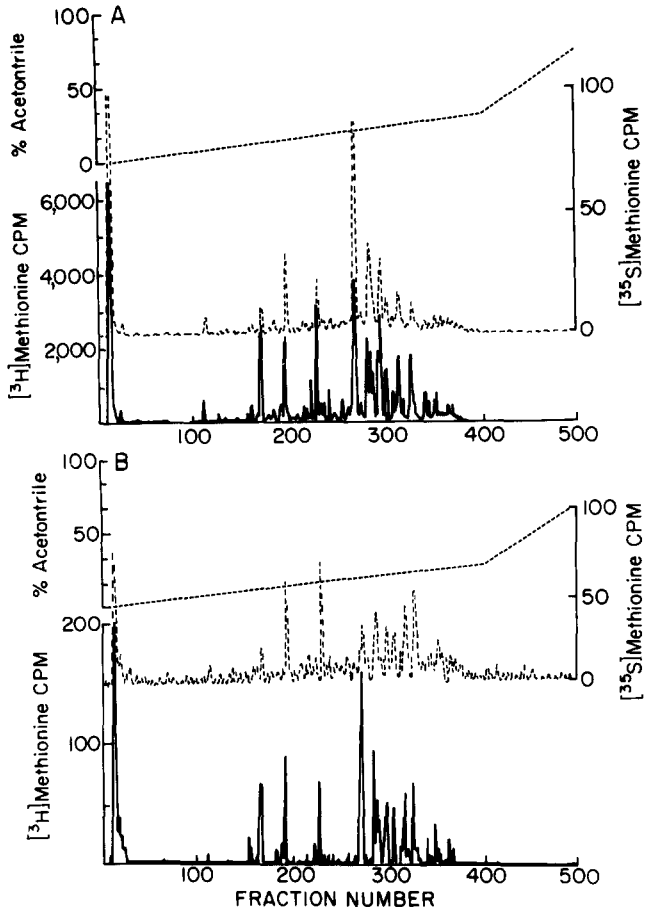

FIGURE 4. Comparative tryptic peptide maps of the CSPG core protein and invariant chain. Gel pieces containing the [^{35}S]methionine-labeled CSPG core protein (*dashed line*) and the corresponding [^{3}H]methionine labeled conventional invariant chain species (*solid line*) were excised from the slab gel shown in Fig. 1, and combined, and the proteins digested with trypsin. The resulting tryptic peptides were chromatographed on a C-18 reverse-phase column using a gradient of 0–50% acetonitrile in 0.1% trifluoracetic acid (-------). (*A*) Comparison of the 38,000 M_r core protein and γ_1 (Fig. 1, *arrows*). (*B*) Comparison of the 18,000 M_r core protein and p25 (Fig. 1, *arrowheads*).

invariant chain (Fig. 2, lanes *5* and *6*, *arrowheads*) were excised and combined. The two sets of samples were subjected to trypsin digestion, and the resultant peptides were separated on a C-18 reverse-phase HPLC column using a gradient of 0–50% acetonitrile in 0.1% trifluoroacetic acid. The elution profiles are shown in Fig. 4. The analysis of the larger M_r proteins is shown in Fig. 4*A*. 9–10 peptides are seen for each chain. Although there are some quantitative differences, virtually all of the peptides coelute, indicating the nearly complete structural identity of the major CSPG core protein and invariant chain. The analysis of the smaller-M_r set of proteins is seen in Fig. 4*B*. Again, nine peptides are observed for each chain, and virtually all of the peptides coelute. These results demonstrate the structural relatedness of these components. The M_r

differences observed on one- and two-dimensional gels between the CSPG core proteins and the invariant chain proteins 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.

Comparison of the profiles in Fig. 4*A* and *B* demonstrates correspondence of the peptides of p25 with those of invariant chain (*solid lines*) and of the peptides of the 28,000 M_r core protein with those of the 38,000 M_r core protein (*dashed lines*). These results indicate that in each case, the smaller-M_r species is clearly related to the larger-M_r species. It is unclear if the smaller species is a partial degradation product of the larger, or is an alternatively processed form. In addition, a peptide map of p41 (data not shown) demonstrated its relatedness to p25 and γ_1.

The experiments above demonstrated the structural relatedness of the core protein(s) and invariant chain protein(s). We next determined whether these proteins were antigenically similar. A [^{35}S]methionine-labeled, doubly purified CSPG preparation was further purified by an additional passage over DEAE-Sephacel under dissociative conditions to eliminate any possible contamination of the CSPG preparation with conventional invariant chain. Aliquots of a doubly purified $^{35}SO_4$-labeled CSPG preparation and the triply purified [^{35}S]methionine-labeled CSPG preparation were taken to test for reactivity with anti–invariant chain antibody. Half of the [^{35}S]methionine-labeled preparation was treated with chondroitinase to generate the core proteins. The samples were then subjected to a pre-precipitation step with RαMIg–protein A to remove any radiolabeled material which potentially could bind nonspecifically to the immunoprecipitates. The samples were then divided into aliquots and tested for reactivity with D1C10, VIC-Y1, C351, and NRS. The results are shown in Figs. 5 and 6. The $^{35}SO_4$-labeled CSPG (Fig. 5), the [^{35}S]methionine labeled CSPG (Fig. 6*A*), and the [^{35}S]methionine-labeled core proteins (Fig. 6*B*) were not isolated in the D1C10 or NRS immunoprecipitates, but were isolated with the VIC-Y1 and C351 anti–invariant chain reagents. These results indicated that the CSPG and its core protein bore epitopes recognized by anti–invariant chain antibodies. Of note, the VIC-Y1 antibody made against intact human invariant chain reacts far better with the 38,000 M_r core protein than with the 28,000 M_r species (Fig. 6*A*, lane *VIC-Y1*). In contrast, the C351 and antibody made against the *C*-terminal region synthetic peptide reacts with both the 38,000 and 28,000 M_r core protein species in proportion to their representation in the eluate. (Fig. 6*A*, lane *351*). This finding suggests that the 28,000 M_r species includes at least some of the amino acid residues present in the synthetic peptide (residues 192–211), but either does not have or does not efficiently present the epitope recognized by VIC-Y1.

The absence of any conventional invariant chain in the [^{35}S]methionine labeled CSPG preparation is demonstrated in Fig. 6*A*, lane *1* (eluate) and is confirmed by the two-dimensional gel analysis of a doubly purified CSPG preparation shown in Fig. 3*A*. This finding indicates that the presence of the CSPG and its core proteins in the anti–invariant chain immunoprecipitates in this reimmunoprecipitation experiment is attributable to a direct reaction of these molecules with the

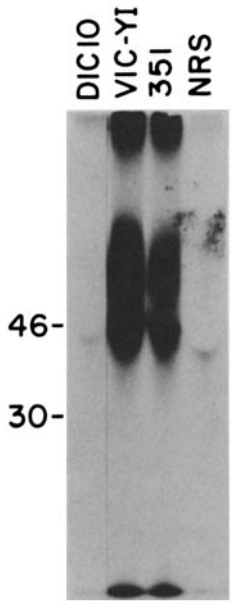

FIGURE 5. $^{35}SO_4$-labeled CSPG can be reprecipitated with anti-invariant chain antibody. Doubly purified $^{35}SO_4$-labeled CSPG was prepared from Swei cells as described in Materials and Methods. The $^{35}SO_4$-labeled CSPG was precleared with RαMIg and protein A, and then aliquots were reacted with D1C10, VICY1, C351, or NRS, and immunoprecipitated with RαMIg–protein A or protein A.

antibody, and not to a possible association of these molecules with any invariant chain that potentially could have been contaminating the triply purified samples.

The CSPG purified from class II immunoprecipitates prepared using VIC-Y1 and the CSPG purified from class II immunoprecipitates prepared using 1800D2.4 have superimposable one- and two-dimensional gel patterns, indicating their identity (data not shown). For quantitative reasons, the [^{35}S]methionine-labeled CSPG purified from the VIC-Y1 immunoprecipitate was used in the preceding experiments. Identical results to those shown in Fig. 5 were obtained with an $^{35}SO_4$-labeled CSPG purified from an 1800D2.4 immunoprecipitate (data not shown). The results from the preceding experiments confirm the structural and antigenic identity of the human CSPG core protein and invariant chain.

In order to gain insight into a potential role of the CSPG in the biology of the class II molecules, we next addressed the question of where in the cell the CSPG–class II complex was located. Because of the known role of proteoglycans in intercellular (15) and cell matrix (16) interactions, we first determined whether the proteoglycan was associated with class II molecules at the cell surface. Intact cells were radiolabeled with [^{3}H]leucine or $^{35}SO_4$ for 5 h. After washing, the cells were divided into two aliquots. To detect cell surface class II molecules, one aliquot was reacted directly with antibody for 30 min, and then lysed in PBS containing 0.5% NP-40 and a fivefold excess of unlabeled cells to bind any free remaining antibody sites. To detect total cellular class II molecules, the second aliquot of cells was lysed in PBS containing 0.5% NP-40, processed in the usual

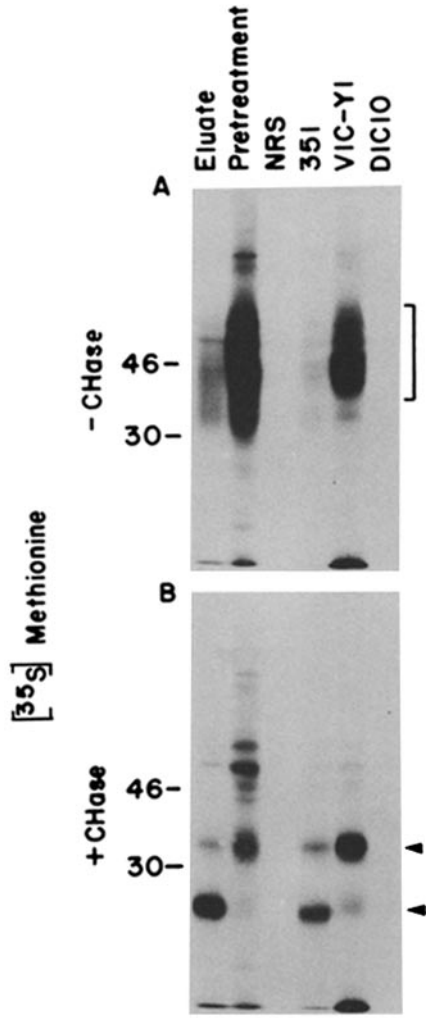

FIGURE 6. [^{35}S]methionine-labeled CSPG and its core proteins can be reprecipitated with anti–invariant chain antibody. Aliquots of a triply purified [^{35}S]methionine-labeled CSPG (*A*) and its core proteins (*B*) were used to test reprecipitation with anti–invariant antibodies. The samples were precleared, and reacted with NRS, C351, YIC-Y1, and D1C10. Immunoprecipitates were made with protein A–Sepharose alone or with protein A–Sepharose armed with RαMIg. The *bracket* in *A* shows the area of the gel in which [^{35}S]methionine-labeled core protein is found. The arrowheads in *B* indicate the 38,000 and 28,000 M_r core proteins. The gel patterns of the eluate after pretreatment and the pretreatment immunoprecipitate are shown for comparison. Abbreviation: CHase, chondroitinase.

way, and then reacted with antibody. Immunoprecipitates were prepared using RαMIg-SaCI, and analyzed by SDS-PAGE. The results are shown in Fig. 7. [^{3}H]leucine-labeled class II molecules (*top*) are detected both from the total cell lysates (lane *DR*) and at the cell surface (lane *DR*). In addition, immunoprecipitates prepared after reaction of the antibody with cell surface class II molecules clearly contain $^{35}SO_4$-labeled CSPG (Fig. 7, *bottom*, lane *DR*). In each case, more material is immunoprecipitable from the cell lysates than from the cell surface.

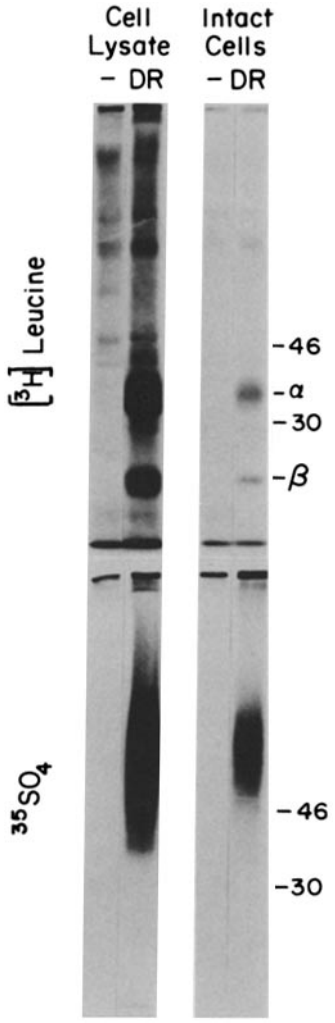

FIGURE 7. The CSPG is associated with HLA class II molecules of the cell surface. Swei cells were labeled with [^{3}H]leucine (*top*) or $^{35}SO_4$ (*bottom*). After labeling, immunoprecipitates were prepared either from NP-40 lysates (*left lanes*) or by incubation of intact cells with antibody for 30 min at 4°C prior to detergent solubilization (*right lanes*) using control (−) or DR antibodies. The running gel is a 10% polyacrylamide gel.

These results indicate that the CSPG is at least in part associated with cell surface class II molecules. Of note, similar results were obtained after only a 15-min pulse label (no chase) of $^{35}SO_4$ indicating that this association of $^{35}SO_4$-labeled CSPG with cell surface Ia is extremely rapid. However, these results do not directly determine whether the CSPG itself is expressed at the cell surface.

To address this last question, cells were labeled with [^{3}H]leucine or $^{35}SO_4$ for 3 h, washed, and then divided into two aliquots. One aliquot of intact cells was treated with chondroitinase AC to remove all cell surface proteoglycan, and the second aliquot was mock digested. Cell viability after chondroitinase digestion was >98%. After digestion, the cells were washed three times, and solubilized in

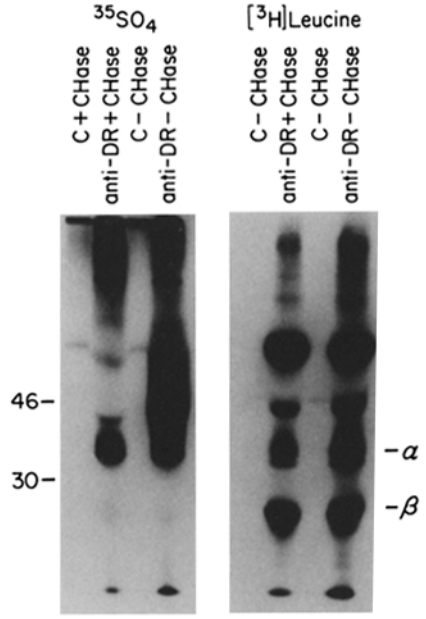

FIGURE 8. The class II–associated CSPG is on the cell surface. Swei cells were labeled with $^{35}SO_4$ (*left lanes*) or [^{3}H]leucine (*right lanes*) and washed extensively. Samples of labeled intact cells were divided in half and either digested with chondroitinase (+ CHase) or mock digested (−CHase). The cells were again washed extensively and solubilized with NP-40, and the lysates reacted the control D1C10 (*C*) or 1800D2.4 (anti-DR) monoclonal antibodies. The [^{3}H]-leucine-labeled α and β chains are seen in both the chondroitinase-treated and mock-digested samples. In contrast, the $^{35}SO_4$-labeled proteoglycan is seen only in the mock-digested samples. The residual $^{35}SO_4$-labeled band seen after chondroitinase treatment is the sulfated α chain.

PBS containing 0.5% NP-40. The processed lysates were reacted with D1C10 and 1800D2.4. The SDS-PAGE analysis of immunoprecipitates is shown in Fig. 8. Equal amounts of [^{3}H]leucine-labeled DR molecules are precipitable from both the chondroitinase- and mock-digested samples. In contrast, $^{35}SO_4$-labeled CSPG is present in the sample prepared from mock-digested intact cells, but totally absent from the sample prepared from chondroitinase-treated intact cells. The residual $^{35}SO_4$-labeled band seen after chondroitinase treatment is sulfated α chain.[3] These results indicate that virtually all the $^{35}SO_4$-labeled class II–associated CSPG is accessible to chondroitinase, and therefore must be expressed on the cell surface.

One caveat in the above experiment is the possibility that even after extensive washing, chondroitinase remained on the intact cells. Solubilization by NP-40 would then allow intracellular proteoglycans to be digested by the enzyme, and any intracellular class II–associated CSPG would no longer be detectable. To address this possibility, unlabeled intact Swei cells were either chondroitinase treated or mock digested, and washed three times to remove the enzyme. An equal number of $^{35}SO_4$-labeled intact Swei cells was then added to each aliquot of unlabeled cells, the mixtures were solubilized by NP-40 detergent, and

[3] Sant, A. J., M. Zacheis, T. Rumbarger, K. S. Giacoletto, and B. D. Schwartz. Human Ia α and β chains are sulfated. Manuscript submitted for publication.

incubated at 37°C for 30 min. Chondroitinase was added back to one aliquot of the lysate of $^{35}SO_4$-labeled and mock-digested unlabeled cells, and the mixture was incubated at 37°C for 30 min. The processed lysates were then reacted with 1800D2.4 or D1C10. Equal amounts of $^{35}SO_4$-labeled CSPG were precipitated from the lysates that contained the condroitinase-treated or mock-digested unlabeled cells (data not shown). In contrast, no $^{35}SO_4$-labeled material was detected when chondroitinase had been added after solubilization. These results indicate that chondroitinase is not being retained on the intact cells after washing, and confirm the interpretation of the results of the previous experiment that virtually all the class II–associated CSPG is present on the cell surface.

Discussion

The experiments presented here indicate that the human invariant chain (γ_1) and its related components are the core proteins of the human class II–associated CSPG. They thus establish that in humans, as in mice (7), the CSPG is an alternatively processed form of variant chain. Further, the results indicate that virtually all the class II–associated CSPG is present on the cell surface.

The purification method we used was modified from that used in the murine system (7), and involved three steps: associative affinity chromatography, immunoprecipitation, and dissociative affinity chromatography. This procedure resulted in a >7,000-fold purification of the class II–associated CSPG, based on [^{35}S]methionine radioactivity. Removal of the glycosaminoglycan yielded the core proteins.

On one-dimensional gel analysis, three distinct core proteins were visualized. These migrated with M_r of 46,000, 38,000, and 28,000, and had the same periodicity as the p41, γ_1, and p25 invariant chain proteins. Tryptic peptide analysis confirmed the identity of the 38,000 M_r core protein and γ_1, and of the 28,000 M_r core protein and p25. Although there was insufficient radioactivity of the 46,000 M_r core protein to prove identity of the 46,000 M_r core protein and p41 by tryptic peptide analysis, we believe that this is most likely the case. In addition, the peptide maps indicated the relatedness of p41, γ_1, and p25, and of the 38,000 and 28,000 M_r core proteins. These results suggest at least two possibilities for the finding of several core proteins. First, each invariant chain species can be independently processed to a proteoglycan form. The individual invariant chain species may be generated either from distinct transcripts of a single invariant chain gene (17) or as the result of proteolytic degradation of a larger invariant chain species. Alternatively, only the larger forms of invariant chain (i.e., γ_1, γ_2, γ_3 and p41) may be processed to a proteoglycan form, and then this proteoglycan is degraded to a smaller form of which the core protein is 28,000 M_r. Our data do not allow us to distinguish between these possibilities. However, either of these possibilities would account for the finding of two distinct populations of class II–associated proteoglycan on two-dimensional gel electrophoretograms.

Studies on other CSPG suggest that the attachment site of the chondroitin-sulfate glycosaminoglycan is on a serine residue that is immediately succeeded by a glycine residue (18). If this holds true for the class II–associated CSPG, it would place the attachment site for the glycosaminoglycan at serine 202, the

only serine in the human invariant chain sequence followed by glycine. This postulate would then suggest that each core protein bears only a single glycosaminoglycan, though the latter can vary in size. The cleavage of the 38,000 M_r core protein to the 28,000 M_r core protein (and presumably γ_1, to p25) would have to be either *C*-terminal to serine 202 or at the *N* terminus.

The derived amino acid sequence of invariant chain suggests it is a transmembrane protein with the *C* terminus on the outside of the cell (19, 20). The attachment site at serine 202 would thus indicate the glycosaminoglycan is exposed on the cell surface. Our experiments demonstrating that the class II–associated CSPG present in intact cells is susceptible to chondroitinase digestion prove this hypothesis. The result of this experiment was somewhat surprising to us, however, in that it demonstrated that virtually all the class II–associated CSPG in the cell was exposed on the cell surface. This result suggests that the addition of the glycosaminoglycan to the invariant chain core protein must occur immediately prior to the transport of the CSPG to the plasma membrane. It also indicates that at least this form of invariant chain is on the cell surface.

Although some previous reports (4, 21–23) have noted the inability to detect invariant chain on the cell surface, other more recent reports (D. M. O'Sullivan, unpublished data, and 24) have indicated that cell-surface invariant chain can be detected. The ability to detect invariant chain on the cell surface undoubtedly depends on the ability of the antibody used to recognize an available epitope. If the CSPG is the only form of invariant chain present on the cell surface, the availability of the epitope may also be influenced by the presence of the glycosaminoglycan.

The presence of the CSPG form of invariant chain on the cell surface with class II molecules again raises questions regarding the function of the invariant chain/CSPG in the biology of class II molecules. In a previous report (7), we organized the potential functions of this molecule into three non–mutually exclusive areas: biosynthesis/targeting, turnover/recycling, and participation in class II–mediated intercellular events. The data in this and other recent reports do not at this time allow differentiation among these possibilities, but lend some support to each of them. Recent studies from one of our (25) and other (26) laboratories have demonstrated that chloroquine and monensin treatment of cells prevent the dissociation of the invariant chain from the $\alpha\beta$ heterodimer. These data were interpreted to suggest that a low pH processing step, presumably in a *trans* Golgi vesicle is responsible for the dissociation of the invariant chain from the $\alpha\beta$ heterodimer. Disruption of this step may therefore prevent the presumed final step in transport of the $\alpha\beta$ heterodimer to the membrane (D. M. O'Sullivan, unpublished data, and 26). However, because these and other lysosomotropic agents also prevent the addition of glycosaminoglycan to core proteins (27, 28, and S. Rosamond, unpublished observations), these data can also be interpreted to suggest that inhibition of the conversion of the invariant chain to its proteoglycan form prevents the dissociation of the invariant chain from the $\alpha\beta$ complex, and possibly therefore the transport of the $\alpha\beta$ heterodimer to the cell surface by the CSPG. If this latter interpretation is correct, it supports the role of the CSPG in targeting newly synthesized class II molecules to the plasma membrane. Additionally, if recycling of class II molecules is required for

antigen processing and/or presentation, the CSPG could function to target recycled class II molecules back to the cell membrane. In this context, the structural and possible functional parallels between the invariant chain/CSPG and transferrin receptor have already been noted (7).

The third possibility, that the class II–associated CSPG may participate in cellular interactions involving the immune response, receives support from the precedent for such participation by proteoglycans in other systems (15, 16), and from our finding that virtually all the class II–associated CSPG is on the cell surface.

Transfection experiments are currently exploring the role of the invariant chain/CSPG in the expression and function of the class II molecules. These experiments may help differentiate among the potential roles of the invariant chain/CSPG, and should determine if this role is obligatory or facilitating in the biology of class II molecules.

Summary

The human class II–associated chondroitin sulfate proteoglycan (CSPG) was analyzed biochemically and immunologically to determine a possible relationship with the human invariant chain (γ_1) and its related components. The CSPG was purified by a three-step procedure involving associative ion-exchange chromatography, immunoprecipitation, and dissociative ion-exchange chromatography. Treatment of the CSPG with chondroitinase revealed core proteins of M_r ~46,000, 38,000, and 28,000, with the 38,000 species most highly represented. Tryptic peptide analysis revealed identity of the peptides of the 38,000 M_r core protein and γ_1, and of the 28,000 M_r species and p25. The CSPG and its core proteins were shown to react directly with the mouse anti–human invariant chain monoclonal antibody VIC-Y1 and a rabbit antiserum produced against a synthetic peptide corresponding to the *C*-terminal end of invariant chain. These results demonstrate that the invariant chain is the core protein of the class II–associated CSPG. In addition, virtually all the CSPG was shown to be present on the cell surface.

The authors thank Ms. Pat Stewart and Ms. Jewel Aldridge for expert preparation of the manuscript.

Received for publication 2 June 1986 and in revised form 9 July 1986.

References

1. 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.
2. Lloyd, K. O., J. Ng, and W. G. Dippold. 1981. Analysis of the biosynthesis of HLA-DR glycoproteins in human malignant melanoma lines. *J. Immunol.* 126:2408.
3. Charron, D. J., and H. O. McDevitt. 1980. Characterization of HLA-D region antigens by two-dimensional gel electrophoresis. *J. Exp. Med.* 152:85.
4. Kvist, S., K. Wiman, L. Claesson, P. A. Peterson, and B. Dobberstein. 1982. Membrane insertion and oligomeric assembly of HLA-DR histocompatibility antigens. *Cell.* 29:61.

5. Quaranta, V., O. Jajdic, G. Stingl, K. Liszka, M. Monigsmann, and W. Knapp. 1984. A human Ia cytoplasmic determinant located on multiple forms of invariant chain (γ,γ2,γ3). *J. Immunol.* 132:1900.
6. 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.
7. Sant, A. J., S. E. Cullen, K. S. Giacoletto, and B. D. Schwartz. 1985. Invariant chain is the core protein of the Ia-associated chondroitin sulfate proteoglycan. *J. Exp. Med.* 162:1916.
8. Hansen, J. A., S. M. Fu, P. Antonelli, M. Kamuin, J. H. Hurley, R. J. Winchester, B. Dupont, and H. G. Kunkel. 1979. B-lymphoid cell lines derived from HLA-D homozygous donors. *Immunogenetics.* 8:51.
9. Chen, Y. X., R. L. Evans, M. S. Pollack, L. L. Lanier, J. H. Phillips, C. Rousso, N. L. Warner, and F. M. Brodsky. 1984. Characterization and expression of the HLA-DC antigens defined by anti-Leu 10. *Hum. Immunol.* 10:221.
10. Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680.
11. Maizel, J. V., Jr. 1971. Polyacrylamide gel electrophoresis of viral proteins. *Methods Virol.* 5:179.
12. O'Farrell, P. M. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007.
13. Steinman, R. M., J. M. Silvers, and Z. A. Cohen. 1974. Pinocytosis in fibroblasts. Quantitative studies in vitro. *J. Cell Biol.* 63:949.
14. 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.
15. Kjellen, L., A. Oldberg, and M. Hook. 1980. Cell surface heparan sulfate. Mechanisms of proteoglycan-cell association. *J. Biol. Chem.* 255:10407.
16. Hedman, K., J. Christner, I. Julkunen, and A. Vaheri. 1983. Chondroitin sulfate at the plasma membrane of culture fibroblasts. *J. Cell Biol.* 97:1288.
17. Yamamoto, K., N. Koch, M. Steinmetz, and G. J. Hammerling. 1985. One gene encodes two distinct Ia-associated invariant chains. *J. Immunol.* 134:3461.
18. Bourdon, M. A., A. Oldberg, M. Pierschbacher, and E. Ruoslahti. 1985. Molecular cloning and sequence analysis of a chondroitin sulfate proteoglycan cDNA. *Proc. Natl. Acad. Sci. USA.* 82:1321.
19. 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.
20. Claesson, L., D. Larhammar, L. Rask, 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.
21. Owen, M. J., A. M. Kissonerghis, H. F. Lodish, and M. J. Crumptom. 1981. Biosynthesis and maturation of HLA-DR antigens in vivo. *J. Biol. Chem.* 256:8987.
22. 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.
23. Machamer, C. E., and P. Cresswell. 1982. Biosynthesis and glycosylation of the invariant chain associated with HLA-DR antigens. *J. Immunol.* 129:2564.
24. Elliot, W. L., P. S. Reisert, M. McFadden, C. Kilcoyne, Q. Nguyen, T. Sairenji, R. C. Spiro, B. A. Woda, and R. E. Humphreys. 1986. Class II antigen-independent expression of I_i. *Fed. Proc.* 45:4874.
25. Nowell, J., and V. Quaranta. 1985. Chloroquine affects biosynthesis of Ia molecules

by inhibiting dissociation of invariant (γ) chains from α-β dimers in cells. *J. Exp. Med.* 162:1371.

26. Machamer, C. E., and P. Cresswell. 1984. Monensin prevents terminal glycosylation of the *N*-linked and *O*-linked oligosaccharides of the HLA-DR associated invariant chain and inhibits its dissociation from the $\alpha\beta$ chain complex. *Proc. Natl. Acad. Sci. USA.* 81:1287.
27. Harper, J. R., V. Quaranta, and R. A. Reisfeld. 1986. Ammonium chloride interferes with a distinct step in the biosynthesis and cell surface expression of human melanoma-type chondroitin sulfate proteoglycan. *J. Biol. Chem.* 261:3600.
28. Nishimoto, S. K., T. Kajiwara, P. W. Ledger, and M. L. Tanzer. 1982. Effects of the ionophore monensin on Type II collagen and proteoglycan synthesis and secretion by cultured chondrocytes. *J. Biol. Chem.* 257:11712.